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**14. ABSTRACT:** We investigated whether rexinoids suppress the growth of breast cells, and how they act to inhibit breast cancer development. First, we measured the growth suppressive activity of rexinoids (RO 25-7386, LGD1069 and LG100268) on the growth of breast cells (normal and malignant). Second, we identified the genes induced by rexinoids that could be related to the cell growth inhibition. By MTS assay, we found that all three compounds (Ro25-7386, LGD1069 and LG100268) inhibited significantly the growth of normal breast cells, HMEC, at 10 uM suggesting the chemopreventive property of the rexinoids. The growth of MCF-7 cells was inhibited by Ro25-7386. T47D breast cancer cells were found to be growth suppressive by Ro25-7386 and LGD1069; they strongly suppressed the cell growth by dose-dependent manner. Surprisingly, LGD1069 also induced a mild inhibition (20 % growth inhibition) of MDA-MB-231 at 10 uM suggesting that LGD1069 can inhibit the ER-negative breast cancer cell growth in vitro. Later, we performed the Affymetrix microarray to identify the genes induced by rexinoids. We found several interesting genes induced by the rexinoids.

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# Molecular mechanism by which retinoids prevent breast cancer development

**Identification of biomarkers regulated by the rexinoids (Ro25-7386, LGD1069 and LG100268) in human breast cells using Affymetrix Microarray**

**Final report, June 30, 2007**

**Department of Thoracic/Head and Neck Medical Oncology**
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**Houston, Texas**
**Hye-Sook SEO**

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# INTRODUCTION

We investigated whether rexinoids suppress the growth of breast cells, and how they act to inhibit breast cancer development. First, we measured the growth suppressive activity of rexinoids (RO 25-7386, LGD1069 and LG100268) on the growth of breast cells; normal breast cells (HMEC), ER-positive breast cancer cells (MCF-7 and T47D) and ER-negative breast cancer cells (MDA-MB-231 and MDA-MB-435). Second, we identified the genes induced by rexinoids that could be related to the cell growth inhibition. By MTS assay, we found that all three compounds (Ro25-7386, LGD1069 and LG100268) inhibited significantly the growth of normal breast cells, HMEC, at 10 uM suggesting the chemopreventive property of the rexinoids. The growth of MCF-7 cells was inhibited by Ro25-7386. T47D breast cancer cells were found to be growth suppressive by Ro25-7386 and LGD1069; they strongly suppressed the cell growth by dose-dependent manner. Surprisingly, LGD1069 also induced a mild inhibition (20 % growth inhibition) of MDA-MB-231 at 10 uM suggesting that LGD1069 can inhibit the ER-negative breast cancer cell growth in vitro. Later, we performed the Affymetrix microarray to identify the genes induced by rexinoids (Ro25-7386, LGD1069 and LG100268). We found several interesting genes induced by the rexinoids. (Please see APPENDICES for the journal article which will be submitted shortly).

## BODY and RESULTS

(Please see APPENDICES for the journal article which will be submitted shortly).

To start the grant research, we planned to follow the approved statement of work using the noticed breast cell lines (HMEC, MCF-7, T47D, MDA-MB-231 and MDA-MB-435), using RXR alpha-bound compound, Ro25-7386. We previously focused on LGD1069 and LG100268 compounds (rexinoids) which randomly bind to RXR isoforms and planed to work on LGD1069-target gene IGFBP-6 to investigate the role of this gene in breast cancer suppression induced by LGD1069 (statement of work).

However, from the first period of this study, we used another specific rexinoid, Ro25-7386 which exclusively bind RXR α isoform. Then, we performed newly Affymetrix microarray data for Ro25-7386, and even LGD1069 and LG100268 to find interesting target genes in normal breast cells and breast cancer cells. Since,

1) We could not obtain the compounds, LGD1069 and LG100268 from the Ligand Pharmaceuticals for the first year.

2) IGF binding protein-6 (IGFBP-6) was reported to be inducible by RA (all-*trans*-retinoic acid) in the retinoid resistant cells, BEAS-2B-R1 human bronchial epithelial cells (Ma et al., 2003).

3) RXR α seems to play a critical role in tumor suppression.

4) We submitted the total RNA samples to the microarray after 12 h time point induction; this time point was selected for study since retinoid treatment would likely regulate the expression of genes earlier than 24 h (Li et al., 2000).

First, we measured the growth suppressive activity of each of rexinoids (Ro25-7386, LGD1069 and LG100268) on the growth of breast cells; normal breast cells (HMEC), ER-positive breast cancer cells (MCF-7 and T47D) and ER-negative breast cancer cells (MDA-MB-231 and MDA-MB-435).

By MTS assay, we found that all three compounds (Ro25-7386, LGD1069 and LG100268) inhibited significantly the growth of normal breast cells, HMEC, at 10 uM

suggesting the chemopreventive property of the rexinoids. The growth of MCF-7 cells was inhibited by Ro25-7386. T47D breast cancer cells were found to be growth suppressive by Ro25-7386 and LGD1069; they strongly suppressed the cell growth by dose-dependent manner. Surprisingly, LGD1069 also induced a mild inhibition (20 % growth inhibition) of MDA-MB-231 at 10 uM suggesting that LGD1069 can inhibit the ER-negative breast cancer cell growth in vitro.

Later, we performed the Affymetrix microarray to identify the target genes induced by rexinoids (Ro25-7386, LGD1069 and LG100268) in each cell line which demonstrated the growth suppressive activity. We found several interesting genes induced by the rexinoids. We selected our major genes of interest by referring to PathArt program which demonstrate the relationship between genes by several signaling pathways.

In HMEC, we identified 638 genes up-regulated and 347 genes down-regulated by Ro25-7386 with changes in fold induction greater than 2 fold. 22 genes were strongly up-regulated (more than 10 fold), and 5 genes were strongly down-regulated (more than 4 fold) in expression levels by Ro25-7386 (Table 1A and 1B). Among them, we found several genes which are involved in cell death, cell growth/maintenance, signal transduction and response to stimulus; i.e., cadherin 1, type1, E-cadherin (CDH1), C-terminal binding protein 1 (CtBP1), integrin beta 4, integrin alpha 6, paxillin (PAX), BCL2-associated X protein (BAX), forkhead box O3A (FOXO3A), signal transducer and activator of transcription 3 (STAT3) (up-regulated genes); collagen type VI alpha 3 and cell division cycle 42 (CDC42) (down-regulated genes). In T47D, we found only 3 up-regulated genes and 5 down-regulated genes with changes in fold induction greater than 2 fold. Among them, we found chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha) (up-regulated genes); Glutamate receptor, ionotropic kainite 2, colon carcinoma related protein, insulin-like growth factor binding protein 7 (IGFBP-7), growth differentiation factor 8, KIAA0998. In MCF-7, we identified 83 genes up-regulated and 98 genes down-regulated by Ro25-7328 with changes in fold induction greater than 2 fold. Among them, we found several genes which are involved in cell

death, cell growth/maintenance, signal transduction and response to stimulus; i.e., TGF beta2, PKC delta binding protein, Neurophilin 2, Interleukin 6 receptor (up-regulated genes); TGF beta1, Integrin beta 4., Paxillin (PAX), Tumor protein 53 (TP53), C-terminal binding protein 1 (CtBP-1), Mcl-1, Bcl-like1 (down-regulated genes).

In T47D, 16 up-regulated genes and 3 down-regulated genes modulated by LGD1069; 3 up-regulated genes and 5 down-regulated genes were modulated by LGD100268 with changes in fold induction greater than 2 fold. In MDA-MB-231, we identified 333 genes up-regulated and 319 genes down-regulated by LGD1069 with changes in fold induction greater than 2 fold. We also identified 116 genes up-regulated and 431 genes down-regulated by LG100268 with changes in fold induction greater than 2 fold. According to the data, we found several interesting genes induced by LGD1069 and LG100268 in MDA-MB-231 such as several hypothetical proteins, CDC14 cell division cycle 14 homolog A (S. cerevisiae), recombination activating gene 2, tumor protein D52, MDM2, ITGA4, ADh1B, NF2 and cathepsin S (for LGD1069), several hypothetical proteins, zinc finger protein 423, cyclin-dependent kinase inhibitor 1C (p57, Kip2), eukaryotic translation initiation factor 5A, transcription factor 4, MDM2, FGF2, GNRH1, and annexin A9 (for LG100268). We will study their functions. We are also performing gene profiling for HMEC to identify the genes regulated by LGD 1069 and LG100268 – this step is still on-going.

# KEY RESEARCH ACCOMPLISHMENTS

1. Ro25-7386, LGD1069 and LG100268 strongly suppressed the growth of normal breast cells (HMEC) displaying their potential chemopreventive activity.

2. Ro25-7386 and LGD1069 strongly suppressed the growth of T47D. MDA-MB-231 cells displaying its therapeutic role in ER-positive as well as ER-negative breast cancer.

3. Gene profiling for RXR α-target genes induced by Ro25-7386 in normal HMEC.

4. Gene profiling for RXR α-target genes induced by Ro25-7386 in T47D.

5. Gene profiling for RXR α-target genes induced by Ro25-7386 in MCF-7.

6. Gene profiling for LGD1069 and LG100268 in normal HMEC.

7. Gene profiling for LGD1069 and LG100268 in MCF-7.

8. Gene profiling for LGD1069 and LG100268 in MDA-MB-231.

# REPORTABLE OUTCOMES

1. Some part of this work wais presented in **2005 Era of Hope meeting (poster presentation- #poster number; p36-13 #abstract – proceedings ; pp 253)**

2. Some part of this work was presented **in 2006 AACR International Conference on Frontiers in Cancer Prevention Research (poster presentation- #poster numb**er**; #126)**

3. Some part of this work was presented in **2007 AACR annual meeting (poster presentation- #poster numb**er**; #4420)**

# CONCLUSION

(Please see APPENDICES for the journal article which will be submitted shortly).

We have studied the molecular mechanism by which retinoids suppress breast cancer development. For that purpose, we tested the growth suppressive activity of the rexinoids (Ro25-7386, LGD1069 and LG100268) in human normal and malignant breast cells. Then, we identified biomarkers regulated by the rexinoids in human normal and malignant breast cells using Affymetrix Microarray.

According to our study, it is demonstrated that LGD1069 might be useful for the prevention and treatment of both ER-positive and ER-negative breast cancer. Moreover, LG100268 which is shown its weaker activity compare to LGD1069 should be more chemopreventive than chemotherapeutic.

This study could help us to find new preventive/therapeutic target for breast cancer, and may contribute to develop novel molecule which could inhibit breast cancer development.

Further, we need to confirm the expression levels of mRNA and protein by real-time RT-PCR and western blot analysis. We also need to study the function of selected genes through the use of the recombinant protein and siRNA transfection. This study could help us to find new preventive/therapeutic target for breast cancer, and may contribute to develop novel molecule which could inhibit breast cancer development.

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# APPENDICES

# Identification of biomarkers regulated by the rexinoids (Ro25-7386, LGD1069 and LG100268) in human breast cells using Affymetrix Microarray

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## ABSTRACT

In this study, we identified genes modulated by rexinoids (RXR-pan agonist; LGD1069 and LG100268) and RXR-α agonist (Ro25-7386)-regulated genes by

affymetrix microarray in normal and malignant breast cells. Ro25-7386, LGD1069 and LG100268 suppressed strongly the growth of normal HMEC (human mammary epithelial cells) at 10 uM suggesting the chemopreventive property of the rexinoids. The growth of MCF-7 cells was inhibited by Ro25-7386. T47D breast cancer cells were found to be growth suppressive by Ro25-7386 and LGD1069; they strongly suppressed the cell growth by dose-dependent manner. Surprisingly, LGD1069 also induced a mild inhibition (20 % growth inhibition) of MDA-MB-231 at 10 uM suggesting that LGD1069 can inhibit the ER-negative breast cancer cell growth in vitro.

Later, to identify genes that are regulated by rexinoids-regulated genes, we treated each cell line (HMEC, T47D, MCF-7, MDA-MB-231) with each rexinoid (Ro25-7386, LGD1069 and LG100268) for 12h using the dose which most strongly suppressed the cell growth (10 μM). We then examined changes in gene expression by using the affymetrix microarray (human genome U133A 2.0) to further investigate which genes are related to the cell growth inhibition induced by the rexinoids.

In HMEC, we identified 638 genes up-regulated and 347 genes down-regulated by Ro25-7386 with changes in fold induction greater than 2 fold. In T47D, we found only 3 up-regulated genes and 5 down-regulated genes with changes in fold induction greater than 2 fold when treated with Ro25-7386. In MCF-7, we identified 83 genes up-regulated and 98 genes down-regulated by Ro25-7386 with changes in fold induction greater than 2 fold.

In T47D, 16 up-regulated genes and 3 down-regulated genes modulated by LGD100268 with changes in fold induction greater than 2 fold. In MDA-MB-231, 333 up-regulated genes and 319 down-regulated genes modulated by LGD1069; 118 up-regulated genes and 432 down-regulated genes were modulated by LG100268 with changes in fold induction greater than 2 fold. Among them, we found several genes which are involved in cell death, cell growth/maintenance, signal transduction and response to stimulus. These rexinoid-regulated genes may be associated with growth suppressive activity of Ro25-7386, LGD1069 or LG100268 in breast cells. Also they can be served as biomarkers and new molecular targets for the prevention and treatment for the breast cancer using rexinoids.

# INTRODUCTION

Breast cancer is the most diagnosed cancer in women and the second leading cause of death from cancer in women (1). In 2007, the ACS estimates 178,480 women will be newly diagnosed with breast cancer, and 40,460 women will be died from this disease (2). Ultimate purpose of scientist and clinicians to conquer this disease is to prevent the incidence, detect early and treat breast cancer with effective therapy resulting in long overall survival minimizing side effects. Therefore, the purpose of this study is to find the way to prevent and treat breast cancer identifying the genes related to the cell growth inhibition which are induced by Retinoid X receptor (RXR)-selective retinoids (rexinoids).

Retinoids regulate a variety of biological functions such as embryogenesis, growth, differentiation, vision and reproduction (3-6). Retinoids also contain anti-proliferative properties, demonstrating their chemopreventive and therapeutic role against cancer (7). Moreover, retinoids is known to inhibit normal- or tumor-cell growth through the regulation of differentiation and/or apoptosis (8-11).

The retinoids exert their effects in target cells interacting with RARs and RXRs,. Each of these includes three subtypes, designated $\alpha$, $\beta$ and $\gamma$, which are encoded by distinct genes. The RAR$\alpha$, RAR$\beta$, and RAR$\gamma$ genes have been localized to chromosomes17q21, 3p24 and 12q13, respectively. The RXR$\alpha$, RXR$\beta$, and RXR$\gamma$ genes have been mapped to chromosomes 9q34.3, 6p21.3 and 1q22-23, respectively (12). The RAR bind both ATRA and 9-*cis* RA while RXR bind only 9-*cis* RA. RAR can form

heterodimers with RXR. RXRs are well-known to heterodimerize with several steroid hormone receptors, such as RAR, TR, VDR, PPAR, LXR, PXR and FXR suggesting its involvement in several signaling pathways. RXR also homodimerize in transfected cells (13).

In addition to naturally occurring retinoids, such as all-*trans* retinoic acid (ATRA), 9-*cis* retinoic acid (9-*cis*-RA) and 13-*cis* retinoic acid (13-*cis*-RA), various synthetic retinoids with different selectivity have been developed and are currently available to treat psoriasis, acne, photoaging, actinic keratosis or cancers such as acute promelocytic leukemia, cutaneous T-cell lymphoma, and squamous or basal cell carcinoma (14). However, the use of RAR-selective retinoids is limited by their toxicity which can include chelitis, hypertriglyceridemia and hepatosplenomegaly (15).

Rexinoids are important in controlling apoptosis and can function in a ligand-dependent or ligand-independent manner (16, 17). Importantly, rexinoids are known to suppress both ER-positive and ER-negative mammary tumor development with reduced toxicity compared to RAR-selective retinoids (18-20). Rexinoids are also active in animals in tamoxifen-resistant breast cancer (17, 21) and in ATRA-resistant breast cancer cells (23). Hence, rexinoids seems to be more efficient and promising chemopreventive and therapeutic drugs compare to RAR-selective ligands. Among them, LGD1069 (Bexarotene) was confirmed as safe and well tolerated agent in clinical trials of cutaneous T-cell lymphoma, breast cancer and lung cancer (23-24)

We, therefore, are interested in rexinoid and its cognate receptor, RXR in breast cells studying their regulatory activity in transcription of genes involved in growth suppression. We concentrated our focus on the RXRα isoform which is a potential therapeutic target in breast cancer cells since over-expression of RXRα sensitized breast cancer cells lines to the anti-proliferative effect of RXR-selective ligand (25), moreover, infection of adenoviral RXRα induced nucleoplasmic over-expression of RXRα and resulted in apoptosis under treatment with an RXR ligand in retinoid-resistant MDA-MB-231 (27). Hence, in this study, we tested 1) the growth suppressive activity of RXRα specific ligand, Ro25-7386 in normal human mammary epithelial cells (HMEC) and retinoid-sensitive breast cancer cells (T47D and MCF-7) by MTT assay, 2) then identified genes regulated by RXRα which may be involved in the anti-proliferative activity of Ro25-7386 by Affymetrix microarray. We found several genes which are involved in cell death, cell growth/maintenance, signal transduction and response to stimulus. Further studying the function of those genes, we may seek to clarify the mechanism by which rexinoids suppress breast cancer development.

# Materials and Methods

*Ligands and Reagents*

RXRα-selective ligand, Ro 25-7386 was generous gifts from kindly provided by Roche Bioscience, Palo Alto, CA). This compound was diluted in DMSO, purchased from Sigma Chemical Co. (St Louis, MO) giving a final concentration of 0.1 %. Monoclonal or polyclonal antibodies (mouse or rabbit) containing E-cadherin, FKHRL1, Integrin α6, Integrin β4, Bax, transforming growth factor, beta 2 and cdc42 rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

*Cells and Culture Materials*

Human normal mammary epithelial cells (HMEC) were obtained from Clonetics (San Diego, CA). They were obtained as primary cultures derived from healthy women who had undergone reduction mammoplasties. Cells between passages 10 and 11 were used for experiments. Cells were grown and maintained in mammary epithelial basal medium (MEBM) supplemented 13 mg/ml bovine pituitary extract, 0.5 % serum, 5 μg/ml insulin, 10 ng/ml human recombinant epidermal growth factor (EGF), 0.5 mg/ml hydrocortisone, 50 μg/ml gentamicin, and 50 μg/ml amphotericin-β (Clonetics, San Diego, CA). Cells were maintained in a humidified environment at 37 °C with 5 % $CO_2$ in air.

Four different human breast cancer cells (MCF-7, T47D, MDA-MB-231 and MBA-MB-435 cells) purchased from ATCC (Manassas, VA) were grown and maintained in appropriate growth media; minimal essential medium (MEM) for MCF-7, RPMI 1740 for

T47D, MDA-MB-231 and MBA-MB-435 with phenol red (In Vitrogen™, Life Technologies: Carlsbad, CA) supplemented with 10% heat-inactivated FBS. L-glutamine, penicillin, streptomycin and gentamicin were used at the usual concentrations. For all experiments, breast cancer cells were harvested by trypsinization (0.25% trypsin - 0.02% EDTA), seeded and grown in the appropriate media containing 10% FBS in a humidified 95% air 5% $CO_2$ atmosphere.

***Cell growth rate measurements***

The CellTiter 96® $AQ_{ueous}$ Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) was used for the measurement of cell growth rate in breast cancer cells according to the manufactory's protocol. The CellTiter 96® $AQ_{ueous}$ Assay is composed of solutions of a novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine methosulfate; PMS). Briefly, HMEC, T-47D and MCF-7 (each cell line-1,000 cells/well), were plated in 96-multi plastic wells in MEBM (see above). The next day, Ro 25-7386, LGD1069 and LG100268 were added in the growth media, and cell culture was pursued for the following days. Each testing day, MTS were added to the cells, and further incubation was pursued for 2 h. MTS is bioreduced by cells into a formazan product that is soluble in tissue culture medium. The quantity of formazan product as measured by the amount of 490nm absorbance. Each data point was performed in quadruplet and the results were reported as mean absorption (optic density, OD)

***Real-time RT-PCR analysis***

HMEC and MCF-7 cells were cultured until 80%-90% confluence in MEBM. Total RNA was prepared by using the Qiagen RNeasy mini kit (Qiagen, Maryland). cDNA of the 10 selected genes and an internal reference gene (glyceraldehydes-3-phosphate dehydrogenase-GAPDH) was made from each sample, then quantified using a fluorescence-based real-time detection method [Bio-Rad iCycler (Hercules, CA)]. RT-PCR analysis was performed by the standard methods recommended by the RT-PCR kit supplier (Applied Biosystems, Foster City, CA). Primers sequences used for detection of the level of CREB were described in Table 5. For endogeneous control, human GAPDH labeled with VIC$^{TM}$ dye – provided by the company was used. The amplification conditions were as follows: 30 s at 95 °C, 3 min at 95 °C, and 30 s at 95 °C and 60 s at 65 °C for 40 cycles, followed by a final extension for 20 min at 72 °C. The ratio between the values obtained provided relative gene expression levels.

***Western Blot analysis***

HMEC, retinoid-sensitive breast cancer cells (T47D and MCF-7), and retinoid-insensitive breast cancer cell lines (MDA-MB-231 and MDA-MB-435) are cultured in culture medium. Whole cell extracts of each cell line were prepared using 1 X sodium dodecyl sulfate (SDS) Laemmlli lysis buffer (125 mM Tris-HCl pH6.8, 1% SDS, 2% β-mercaptoethanol, 0.01%). Total cell lysates with equal amounts of protein (10 μg) were subjected to 10% SDS-PAGE and subsequently electrotransferred onto a nitrocellulose membrane (Bio-Rad laboratories, Hercules, CA). Membranes were blocked with 5% skim milk in PBST (PBS containing 0.1% Tween20) for 1 h at room temperature then were

incubated overnight with primary antibody in PBST containing 2.5% BSA (1:1000 dilution). After washing with PBST, the blot was further incubated for 1 h at room temperature with peroxidase conjugated anti-rabbit or anti-mouse antibodies (Pierce Technology Corp., NY) in PBST and then visualized by using the ECL system (Amersham Biosciences, Arlington Heights, IL). Protein expression was normalized using beta-actin expression.

# Results

### *Expression of RXRα in breast cells*

In Figure 1, the structure of LGD1069 and LG100268 are displayed. We first determined the expression level of RXRα in 1 normal breast cells (HMEC), two retinoid-sensitive breast cancer cells (MCF-7 and T47D) and two retinoid-insensitive breast cancer cells (MDA-MB-231 and MDA-MB-435). We found that all breast cell lines express RXRα but with different intensity. MCF-7 and T47D expressed higher amount of RXRα (Figure 2). Interestingly, ER-negative breast cancer cell lines which do not respond to retinoid treatment such as MDA-MB-231 and MDA-MB-435 also expressed RXRα.

### *Anti-proliferative activity of RXRα-specific ligand*

Secondly, we tested the anti-proliferative effect of RXRα specific compound, Ro25-7386 in HMEC. By MTS assay, we found that Ro25-7386 suppressed HMEC normal breast cell growth by dose-dependent manner. We also found that Ro25-7386 suppressed T47D cell growth at 1 μM. Ro25-7386 induced mild suppression of the cell growth in MCF-7 at 1 μM (Figure 3). These results indicate that RXRα agonist, Ro25-7386 suppresses the growth of HMEC and retinoid-sensitive breast cancer cells, T47D and MCF-7. Hence, RXRα seems to play an important role for growth suppression induced by rexinoid in breast cells. Moreover, the amount of RXRα receptor may not be related to the inhibition of breast cell growth induced by Ro25-7386. These results indicate that RXRα agonist, Ro25-7386 suppressed the growth of HMEC and retinoid-

sensitive breast cancer cells, T47D and MCF-7. Hence, RXRα seems to play an important role “at least in part” for growth suppression induced by rexinoid in breast cells. Moreover, the amount of RXRα receptor may not be related to the inhibition of breast cell growth induced by Ro25-7386.

***Anti-proliferative activity of RXRα-specific ligand***

Third, we tested the anti-proliferative effect of LGD1069 and LG100268 in HMEC, retinoid-sensitive breast cancer cells (T47D and MCF-7), and retinoid-insensitive breast cancer cells (MDA-MB-231 and MDA-MB-435). By MTS assay, we found that Ro25-7386 suppressed HMEC normal breast cell growth by dose-dependent manner. We also found that Ro25-7386 suppressed T47D cell growth at 1 μM while Ro25-7386 suppressed T47D cell growth at 1 μM. Ro25-7386 induced mild suppression of the cell growth in MCF-7 at 1 μM (Figure 3).

These results indicate that RXRα agonist, Ro25-7386 suppresses the growth of HMEC and retinoid-sensitive breast cancer cells, T47D and MCF-7. Hence, RXRα seems to play an important role for growth suppression induced by rexinoid in breast cells. Moreover, the amount of RXRα receptor may not be related to the inhibition of breast cell growth induced by Ro25-7386. These results indicate that RXRα agonist, Ro25-7386 suppressed the growth of HMEC and retinoid-sensitive breast cancer cells, T47D and MCF-7. Hence, RXRα seems to play an important role “at least in part” for growth suppression induced by rexinoid in breast cells. Moreover, the amount of RXRα receptor may not be related to the inhibition of breast cell growth induced by Ro25-7386.

***Anti-proliferative activity of LGD1069 and LG100268***

Third, we tested the anti-proliferative effect of LGD1069 and LG100268 in HMEC, retinoid-sensitive breast cancer cells (T47D and MCF-7), and retinoid-insensitive breast cancer cells (MDA-MB-231 and MDA-MB-435). After 8 days of treatment, we found that LGD1069 suppressed the growth of HMEC by dose-dependent manner; by MTS assay, LG100268 suppressed strongly the growth of HMEC at 10 μM (Figure 4). On the other hand, when cells were submitted to the co-treatment of LGD1069 and LG100268, we could not observe the synergistic effect of the ligands, LGD1069 and LG100268.

Both LGD1069 and LG100268 did not affect the cell growth rate of MCF-7 even at the strongest dose, 10 μM. However, T47D displayed its sensitivity to LGD1069 and LG100268. The rexinoids strongly suppressed the cell growth of T47D by dose-dependent manner after 10 days of treatment (Figure 5). Especially, it seems that LGD1069 has stronger activity compare to LG100268 for the cell growth of T47D.

Rexinoid, especially LGD1069 induced a mild inhibition (20 % inhibition) of the cell growth of MDA-MB-231 at the dose of 10 μM (Figure 6). This result indicates that LGD1069 can inhibit the growth of ER-negative breast cancer with therapeutic potency.

By MTS assay, we found that both LGD1069 and LG100268 inhibited significantly normal HMEC cell growth at 10 uM. We also found that LGD1069 strongly suppressed the growth of T47D (ER-positive) by dose-dependent manner. LGD1069 also

induced a mild inhibition of MDA-MB-231 (ER-negative) at 10 uM. MCF-7 and MDA-MB-435 did not have growth suppression by LGD1069 at 10 uM. LG100268 did affect little the cell growth in all 4 breast cancer cell lines suggesting its weak activity compare to LGD1069 (Figure 6).

We also found that LGD1069 strongly suppressed the growth of T47D (ER-positive) by dose-dependent manner. LGD1069 also induced a mild inhibition of MDA-MB-231 (ER-negative) at 10 uM.

***Determine RXRα target genes of normal and malignant breast cells by Affymetrix microarray***

For the next step of the study, we identified genes regulated by RXRα–specific ligand, Ro25-7386 in normal (HMEC) and malignant (T47D and MCF-7) breast cells. Gene expression profiles were established by using Affymetrix microarray (human genome U133A 2.0). In Figure 7, the comparison between cell growth suppressive activity and Affymetrix Microarray data was shown. For that purpose, we treated breast cells with Ro25-7386 using the concentration which most strongly suppressed the cell growth (10 μM), and total RNA sample was harvested after 12h; this time point was selected for study since retinoid treatment would likely regulate the expression of genes earlier than 24h (28). We then examined changes in gene expression by using the microarray to investigate which genes are related to cell growth inhibition induced by the RXRα agonist. In HMEC, we identified 638 genes up-regulated and 347 genes down-regulated by Ro25-7386 with changes in fold induction greater than 2 fold. 22 genes were

strongly up-regulated (more than 10 fold), and 5 genes were strongly down-regulated (more than 4 fold) in expression levels by Ro25-7386 (Table 1A and 1B).

In HMEC, we identified 638 genes up-regulated and 347 genes down-regulated by Ro25-7386 with changes in fold induction greater than 2 fold. 22 genes were strongly up-regulated (more than 10 fold), and 5 genes were strongly down-regulated (more than 4 fold) in expression levels.

Among them, we found several genes which are involved in cell death, cell growth/maintenance, signal transduction and response to stimulus; i.e., cadherin 1, type1, E-cadherin (CDH1), C-terminal binding protein 1 (CtBP1), integrin beta 4, integrin alpha 6, paxillin (PAX), BCL2-associated X protein (BAX), forkhead box O3A (FOXO3A), signal transducer and activator of transcription 3 (STAT3) (up-regulated genes); collagen type VI alpha 3 and cell division cycle 42 (CDC42) (down-regulated genes). In T47D, we found only 3 up-regulated genes and 5 down-regulated genes with changes in fold induction greater than 2 fold. Among them, we found chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha) (up-regulated genes); Glutamate receptor, ionotropic kainite 2, colon carcinoma related protein, insulin-like growth factor binding protein 7 (IGFBP-7), growth differentiation factor 8, KIAA0998 (Table 2A and 2B). In MCF-7, we identified 83 genes up-regulated and 98 genes down-regulated by Ro25-7328 with changes in fold induction greater than 2 fold (Table 3A and 3B). Among them, we found several genes which are involved in cell death, cell growth/maintenance, signal transduction and response to stimulus; i.e., TGF beta2, PKC delta binding protein,

Neurophilin 2, Interleukin 6 receptor (up-regulated genes); TGF beta1, Integrin beta 4., Paxillin (PAX), Tumor protein 53 (TP53), C-terminal binding protein 1 (CtBP-1), Mcl-1, Bcl-like1 (down-regulated genes).

In T47D, 16 up-regulated genes and 3 down-regulated genes modulated by LGD1069 (Table 4A and 4B); 3 up-regulated genes and 5 down-regulated genes were modulated by LGD100268 with changes in fold induction greater than 2 fold (Table 5A and 5B). In MDA-MB-231, 335 up-regulated genes and 320 down-regulated genes modulated by LGD1069 (Table 6A and 6B); 118 up-regulated genes and 432 down-regulated genes were modulated by LGD100268 with changes in fold induction greater than 2 fold (Table 7A and 7B). According to the data, we found several interesting genes induced by LGD1069 and LG100268 in MDA-MB-231 such as several hypothetical proteins, CDC14 cell division cycle 14 homolog A (S. cerevisiae), recombination activating gene 2, tumor protein D52, MDM2, ITGA4, ADh1B, NF2 and cathepsin S (for LGD1069), several hypothetical proteins, zinc finger protein 423, cyclin-dependent kinase inhibitor 1C (p57, Kip2), eukaryotic translation initiation factor 5A, transcription factor 4, MDM2, FGF2, GNRH1, and annexin A9 (for LG100268). We will study their functions.

*Confirmation of the changes of modulation of RXR$\alpha$ target genes of HMEC by real-time RT-PCR and western blot analysis*

The induction of a total of 7 genes by rexinoid (mRNA levels) was confirmed by real-time RT-PCR assays. They include integrin beta 4, integrin alpha 6, E-cadherin

(CDH1), PAX, BAX, FOXO3A and STAT3; up-regulation of those genes by Ro25-7386 was confirmed (Figure 8). The primers for forward and reverse directions were described in Table 8. The changes in fold induction of protein levels of some genes were confirmed by western blot analysis; up-regulation of BAX, E-cadherin, interleukin alpha 6 and the down-regulation of CDC42 were revealed (Figure 9).

Profound investigation of our interesting genes - CDH1, FOXO3A and BAX (HMEC-Ro25-7386), insulin-like growth factor binding protein 7 (IGFBP-7) and growth differentiation factor 8 (T47D-Ro25-7386) and cathepsin S, TGFβ2, basigin, MCL-1 and BCL2L1 (MCF-7-Ro25-7386), CDH1), C-terminal binding protein 1 (CtBP1) may lead us to clarify how RXR α agonist functions to inhibit breast cell growth. This study could help us to find new preventive/therapeutic target for breast cancer, and may contribute to develop novel molecule which could inhibit breast cancer development.

# Discussion

To reach our ultimate goal – study the molecular mechanism by which retinoids suppress breast cancer development, we focused our attention on RXR-specific ligands (rexinoids) which have been reported to suppress breast cancer development with minimal toxicity compare to RAR-specific ligands. We also especially focused our study toward RXRα isoform, which plays an important role in tumor suppression.

Human RXRα gene spans over 40 kilobases in size and consists of at least 10 exons separated by introns ranging in size from 700 base pairs (intron 3) to more than 7.8 kb (intron 4) (28). We observed that all cell lines tested expressed RXRα. Interestingly, ER-negative breast cancer cells which do not respond to retinoid treatment such as MDA-MB-231 and MDA-MB-435 also expressed RXRα. This suggests that RXRα is non-functional losing DNA binding activity or failing to recruit essential co-activators required for the gene activation in retinoid-insensitive cells. Different and inappropriate sub-localization of the receptor may also explain the unresponsiveness of the cells to retinoid.

We found that LGD1069, LG100268 and RXR α agonist, Ro25-7386 suppressed the growth of breast cells - normal HMEC cells and retinoid-sensitive breast cancer cell lines (MCF-7 and T47D). We found that LGD1069, LG100268 and RXRα agonist Ro25-7386 suppressed the growth of breast cells – normal HMEC cells and retinoid-sensitive breast cancer cell line, T47D. MCF-7 breast cancer cell line was shown as growth

suppressive for Ro25-7386 but was not growth suppressive for LGD1069 and LG100268. As expected, Ro25-7386 had no growth suppressive activity on retinoid-insensitive breast cancer cell lines, MDA-MB-231 and MDA-MB-435 suggesting that RXRα existed in those cell lines is non-functional (data not shown). Surprisingly, especially LGD1069 induced a mild inhibition (20 % inhibition) of the cell growth of MDA-MB-231 at the dose of 10 μM. LG100268 did affect little the cell growth compare to LGD1069 in all 4 breast cancer cell lines suggesting its weaker activity. This result indicates that LGD1069 can inhibit the growth of ER-negative breast cancer with therapeutic potency.

We selected our major genes of interest by referring to PathArt program which demonstrate the relationship between genes by several signaling pathways. We then examined changes in gene expression by using the affymetrix microarray (human genome U133A 2.0) to further investigate which genes are related to the cell growth inhibition induced by the rexinoids.

In HMEC, we identified 638 genes up-regulated and 347 genes down-regulated by Ro25-7386 with changes in fold induction greater than 2 fold. 22 genes were strongly up-regulated (more than 10 fold), and 5 genes were strongly down-regulated (more than 4 fold) in expression levels by Ro25-7386 (Table 1A and 1B).

Among them, we found several genes which are involved in cell death, cell growth/maintenance, signal transduction and response to stimulus; i.e., cadherin 1, type1, E-cadherin (CDH1), C-terminal binding protein 1 (CtBP1), integrin beta 4, integrin alpha

6, paxillin (PAX), BCL2-associated X protein (BAX), forkhead box O3A (FOXO3A), signal transducer and activator of transcription 3 (STAT3) (up-regulated genes); collagen type VI alpha 3 and cell division cycle 42 (CDC42) (down-regulated genes). In T47D, we found only 3 up-regulated genes and 5 down-regulated genes with changes in fold induction greater than 2 fold. Among them, we found chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha) (up-regulated genes); Glutamate receptor, ionotropic kainite 2, colon carcinoma related protein, insulin-like growth factor binding protein 7 (IGFBP-7), growth differentiation factor 8, KIAA0998. In MCF-7, we identified 83 genes up-regulated and 98 genes down-regulated by Ro25-7328 with changes in fold induction greater than 2 fold. Among them, we found several genes which are involved in cell death, cell growth/maintenance, signal transduction and response to stimulus; i.e., TGF beta2, PKC delta binding protein, Neurophilin 2, Interleukin 6 receptor (up-regulated genes); TGF beta1, Integrin beta 4., Paxillin (PAX), Tumor protein 53 (TP53), C-terminal binding protein 1 (CtBP-1), Mcl-1, Bcl-like1 (down-regulated genes).

In T47D, 16 up-regulated genes and 3 down-regulated genes modulated by LGD1069; 3 up-regulated genes and 5 down-regulated genes were modulated by LGD100268 with changes in fold induction greater than 2 fold. In MDA-MB-231, 335 up-regulated genes and 320 down-regulated genes modulated by LGD1069; 118 up-regulated genes and 432 down-regulated genes were modulated by LGD100268 with changes in fold induction greater than 2 fold. According to the data, we found several interesting genes induced by LGD1069 and LG100268 in MDA-MB-231 such as several

hypothetical proteins, CDC14 cell division cycle 14 homolog A (S. cerevisiae), recombination activating gene 2, tumor protein D52, MDM2, ITGA4, ADh1B, NF2 and cathepsin S (for LGD1069), several hypothetical proteins, zinc finger protein 423, cyclin-dependent kinase inhibitor 1C (p57, Kip2), eukaryotic translation initiation factor 5A, transcription factor 4, MDM2, FGF2, GNRH1, and annexin A9 (for LG100268). We will study their functions.

This study could help us to find new preventive/therapeutic target for breast cancer, and may contribute to develop novel molecule which could inhibit breast cancer development.

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**Table 1A. Genes Up-regulated by Ro25-7386 in HMEC**

| probe set | gene | fold change |
|---|---|---|
| 213872_at | gb:BE465032 /DB_XREF=gi:9510807 /DB_XREF=hv76g09.x1 /CLONE=IMAGE:3179392 /FEA=EST /CNT=34 /TID=Hs.173685.1 /TIER=Stack /STK=15 /UG=Hs.173685 /LL=81688 /UG_GENE=FLJ12619 /UG_TITLE=hypothetical protein FLJ12619 | 27.55 |
| 204989_s_at | integrin, beta 4 | 26.6 |
| 210317_s_at | tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide | 22.14 |
| 200935_at | calreticulin | 21.26 |
| 201130_s_at | cadherin 1, type 1, E-cadherin (epithelial) | 20.66 |
| 201123_s_at | eukaryotic translation initiation factor 5A | 19.04 |
| 200751_s_at | heterogeneous nuclear ribonucleoprotein C (C1/C2) | 18.97 |
| 214007_s_at | PTK9 protein tyrosine kinase 9 | 17.37 |
| 203392_s_at | C-terminal binding protein 1 | 16.35 |
| 204427_s_at | coated vesicle membrane protein | 16 |
| 216971_s_at | plectin 1, intermediate filament binding protein 500kDa | 15.17 |
| 217211_at | Consensus includes gb:D50604 /DEF=Human beta-cytoplasmic actin (ACTBP9) pseudogene /FEA=CDS /DB_XREF=gi:2094759 /UG=Hs.248007 Human beta-cytoplasmic actin (ACTBP9) pseudogene | 14.35 |
| 215780_s_at | SET translocation (myeloid leukemia-associated) | 12.51 |
| 201971_s_at | ATPase, H+ transporting, lysosomal 70kDa, V1 subunit A | 11.75 |
| 204426_at | coated vesicle membrane protein | 11.74 |
| 220494_s_at | gb:NM_018678.1 /DEF=Homo sapiens lipopolysaccharide specific response-68 protein (LSR68), mRNA. /FEA=mRNA /GEN=LSR68 /PROD=lipopolysaccharide specific response-68 protein /DB_XREF=gi:8923914 /UG=Hs.103189 lipopolysaccharide specific response-68 protein / | 11.18 |
| 215177_s_at | integrin, alpha 6 | 10.97 |
| 215434_x_at | AG1 | 10.35 |
| 214693_x_at | hypothetical protein MGC8902 /// AG1 /// hypothetical protein DJ328E19.C1.1 /// hypothetical protein LOC200030 /// hypothetical protein LOC348482 | 10.34 |
| 211905_s_at | integrin, beta 4 | 10.34 |
| 201048_x_at | RAB6A, member RAS oncogene family | 10.03 |
| 214701_s_at | fibronectin 1 | 10.01 |
| 210092_at | mago-nashi homolog, proliferation-associated (Drosophila) | 9.74 |
| 212107_s_at | DEAH (Asp-Glu-Ala-His) box polypeptide 9 | 9.68 |
| 202118_s_at | copine III | 9.48 |
| 217234_s_at | villin 2 (ezrin) | 9.09 |
| 208853_s_at | calnexin | 7.59 |
| 201742_x_at | splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor) | 7.44 |
| 208750_s_at | ADP-ribosylation factor 1 | 7.31 |
| 203803_at | prenylcysteine oxidase 1 | 7.31 |
| 211162_x_at | stearoyl-CoA desaturase (delta-9-desaturase) | 7.3 |
| 202856_s_at | solute carrier family 16 (monocarboxylic acid transporters), member 3 | 7.26 |
| 200796_s_at | myeloid cell leukemia sequence 1 (BCL2-related) | 7.25 |
| 213606_s_at | Rho GDP dissociation inhibitor (GDI) alpha | 7.25 |
| 201373_at | plectin 1, intermediate filament binding protein 500kDa | 7.19 |
| 208057_s_at | GLI-Kruppel family member GLI2 | 7.04 |
| 217294_s_at | enolase 1, (alpha) | 6.99 |
| 213875_x_at | chromosome 6 open reading frame 62 | 6.93 |
| 91816_f_at | ring finger and KH domain containing 1 | 6.9 |
| 200806_s_at | heat shock 60kDa protein 1 (chaperonin) | 6.69 |
| 214845_s_at | calumenin | 6.66 |
| 211823_s_at | paxillin | 5.75 |
| 206665_s_at | BCL2-like 1 | 5.4 |
| 208637_x_at | actinin, alpha 1 | 5.11 |
| 208677_s_at | basigin (OK blood group) | 4.66 |
| 221499_s_at | syntaxin 16 | 4.16 |
| 209226_s_at | transportin 1 | 3.9 |
| 201752_s_at | adducin 3 (gamma) | 3.9 |
| 200766_at | cathepsin D (lysosomal aspartyl protease) | 3.9 |
| 203085_s_at | transforming growth factor, beta 1 (Camurati-Engelmann disease) | 3.75 |
| 211833_s_at | BCL2-associated X protein | 3.65 |
| 208852_s_at | calnexin | 3.49 |
| 210655_s_at | forkhead box O3A | 3.33 |

## Table 1B. Genes Down-regulated by Ro25-7386 in HMEC

| probe set | gene | fold change |
|---|---|---|
| 203991_s_at | ubiquitously transcribed tetratricopeptide repeat, X chromosome | -5.32 |
| 220568_at | gb:NM_018582.1 /DEF=Homo sapiens hypothetical protein PRO1483 (PRO1483), mRNA. /FEA=mRNA /GEN=PRO1483 /PROD=hypothetical protein PRO1483 /DB_XREF=gi:8924047 /UG=Hs.279694 hypothetical protein PRO1483 /FL=gb:AF116635.1 gb:NM_018582.1 | -4.72 |
| 213705_at | Methionine adenosyltransferase II, alpha | -4.64 |
| 201438_at | collagen, type VI, alpha 3 | -4.59 |
| 217665_at | Consensus includes gb:AA420614 /FEA=EST /DB_XREF=gi:2094586 /DB_XREF=est:nc62g02.r1 /CLONE=IMAGE:745874 /UG=Hs.188826 ESTs, Moderately similar to G02654 ribosomal protein L39 H.sapiens | -4.17 |
| 209459_s_at | 4-aminobutyrate aminotransferase | -3.99 |
| 220992_s_at | chromosome 1 open reading frame 25 /// chromosome 1 open reading frame 25 | -3.81 |
| 222294_s_at | Eukaryotic translation initiation factor 2C, 2 | -3.78 |
| 221995_s_at | Consensus includes gb:BF195165 /FEA=EST /DB_XREF=gi:11081754 /DB_XREF=est:7n16b01.x1 /CLONE=IMAGE:3564624 /UG=Hs.182695 hypothetical protein MGC3243 | -3.71 |
| 215095_at | Esterase D/formylglutathione hydrolase | -3.68 |
| 212675_s_at | KIAA0582 | -3.66 |
| 210187_at | FK506 binding protein 1A, 12kDa | -3.65 |
| 204634_at | NIMA (never in mitosis gene a)-related kinase 4 | -3.59 |
| 203791_at | Dmx-like 1 | -3.53 |
| 205583_s_at | chromosome X open reading frame 45 | -3.53 |
| 218352_at | regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 1 | -3.52 |
| 209788_s_at | type 1 tumor necrosis factor receptor shedding aminopeptidase regulator | -3.48 |
| 212959_s_at | MGC4170 protein | -3.47 |
| 205802_at | transient receptor potential cation channel, subfamily C, member 1 | -3.43 |
| 202732_at | protein kinase (cAMP-dependent, catalytic) inhibitor gamma | -3.4 |
| 202149_at | neural precursor cell expressed, developmentally down-regulated 9 | -3.39 |
| 213225_at | protein phosphatase 1B (formerly 2C), magnesium-dependent, beta isoform | -3.39 |
| 213624_at | sphingomyelin phosphodiesterase, acid-like 3A | -3.39 |
| 207855_s_at | Mid-1-related chloride channel 1 | -3.37 |
| 204415_at | interferon, alpha-inducible protein (clone IFI-6-16) | -3.29 |
| 210017_at | mucosa associated lymphoid tissue lymphoma translocation gene 1 | -3.12 |
| 205420_at | peroxisomal biogenesis factor 7 | -3.05 |
| 219317_at | polymerase (DNA directed) iota | -3.01 |
| 204176_at | kelch-like ECT2 interacting protein | -3 |
| 203741_s_at | adenylate cyclase 7 | -2.95 |
| 205034_at | cyclin E2 | -2.94 |
| 204078_at | synaptonemal complex protein SC65 | -2.9 |
| 203881_s_at | dystrophin (muscular dystrophy, Duchenne and Becker types) | -2.88 |
| 209717_at | ecotropic viral integration site 5 | -2.87 |
| 213473_at | BRCA1 associated protein | -2.86 |
| 215949_x_at | immunoglobulin heavy constant mu | -2.83 |
| 205668_at | lymphocyte antigen 75 | -2.83 |
| 219688_at | Bardet-Biedl syndrome 7 | -2.82 |
| 207845_s_at | anaphase promoting complex subunit 10 | -2.8 |
| 208920_at | sorcin | -2.79 |
| 218002_s_at | chemokine (C-X-C motif) ligand 14 | -2.53 |
| 208727_s_at | cell division cycle 42 (GTP binding protein, 25kDa) | -2.25 |

## Table 2A. Genes Up-regulated by Ro25-7386 in T47D

| probe set | gene | fold change |
|---|---|---|
| 215653_at | Consensus includes gb:AF339805.1 /DEF=Homo sapiens clone IMAGE:248602, mRNA sequence. /FEA=mRNA /DB_XREF=gi:13507343 /UG=Hs.326719 Homo sapiens clone IMAGE:248602, mRNA sequence | 4.74 |
| 215924_at | Consensus includes gb:AK022102.1 /DEF=Homo sapiens cDNA FLJ12040 fis, clone HEMBB1001944. /FEA=mRNA /DB_XREF=gi:10433423 /UG=Hs.296687 Homo sapiens cDNA FLJ12040 fis, clone HEMBB1001944 | 2.87 |
| 204470_at | chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha) | 2.26 |

## Table 2B. Genes Down-regulated by Ro25-7386 in T47D

| probe set | gene | fold change |
|---|---|---|
| 215655_at | Glutamate receptor, ionotropic, kainate 2 | -3.29 |
| 220327_at | colon carcinoma related protein | -2.94 |
| 213910_at | insulin-like growth factor binding protein 7 | -2.66 |
| 207145_a | growth differentiation factor 8 | -2.49 |
| 210806_at | KIAA0998 | -2.3 |

## Table 3A. Genes Up-regulated by Ro25-7386 in MCF-7

| probe set | gene | fold change |
|---|---|---|
| 209909_s_at | transforming growth factor, beta 2 | 4.94 |
| 211430_s_at | immunoglobulin heavy constant gamma 1 (G1m marker) | 3.82 |
| 213010_at | protein kinase C, delta binding protein | 3.76 |
| 63825_at | Abhydrolase domain containing 2 | 3.4 |
| 208993_s_at | peptidyl-prolyl isomerase G (cyclophilin G) | 3.39 |
| 204681_s_at | Rap guanine nucleotide exchange factor (GEF) 5 | 3.26 |
| 213536_s_at | gb:AA910614 /DB_XREF=gi:3049904 /DB_XREF=ok61b04.s1 /CLONE=IMAGE:1518415 /FEA=EST /CNT=42 /TID=Hs.84285.2 /TIER=Stack /STK=12 /UG=Hs.84285 /LL=7329 /UG_GENE=UBE2I /UG_TITLE=ubiquitin-conjugating enzyme E2I (homologous to yeast UBC9) | 3.2 |
| 213087_s_at | Eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein) | 3.09 |
| 217489_s_at | interleukin 6 receptor | 3.07 |
| 205443_at | small nuclear RNA activating complex, polypeptide 1, 43kDa | 3.04 |
| 213747_at | Consensus includes gb:AA047234 /FEA=EST /DB_XREF=gi:1525134 /DB_XREF=est:zf50b09.s1 /CLONE=IMAGE:380345 /UG=Hs.223014 antizyme inhibitor | 2.99 |
| 221815_at | Abhydrolase domain containing 2 | 2.95 |
| 212451_at | KIAA0256 gene product | 2.93 |
| 205363_at | butyrobetaine (gamma), 2-oxoglutarate dioxygenase (gamma-butyrobetaine hydroxylase) 1 | 2.92 |
| 212952_at | Consensus includes gb:AA910371 /FEA=EST /DB_XREF=gi:3049661 /DB_XREF=est:ok83h10.s1 /CLONE=IMAGE:1520611 /UG=Hs.16488 calreticulin | 2.9 |
| 210136_at | Myelin basic protein | 2.88 |
| 214255_at | ATPase, Class V, type 10A | 2.87 |
| 213789_at | Consensus includes gb:N58493 /FEA=EST /DB_XREF=gi:1202383 /DB_XREF=est:yv72d01.s1 /CLONE=IMAGE:248257 /UG=Hs.75105 emopamil-binding protein (sterol isomerase) | 2.86 |
| 217464_at | Consensus includes gb:L48784 /DEF=050 Homo sapiens cDNA /FEA=mRNA /DB_XREF=gi:1066715 /UG=Hs.182426 ribosomal protein S2 | 2.83 |
| 210841_s_at | neuropilin 2 | 2.82 |
| 204378_at | breast carcinoma amplified sequence 1 | 2.8 |
| 208859_s_at | alpha thalassemia/mental retardation syndrome X-linked (RAD54 homolog, S. cerevisiae) | 2.76 |
| 221018_s_at | tudor domain containing 1 /// tudor domain containing 1 | 2.76 |
| 218876_at | brain specific protein /// brain specific protein | 2.73 |
| 215081_at | KIAA1024 protein | 2.71 |
| 201510_at | E74-like factor 3 (ets domain transcription factor, epithelial-specific ) | 2.69 |
| 210089_s_at | laminin, alpha 4 | 2.68 |
| 218859_s_at | chromosome 20 open reading frame 6 | 2.65 |
| 211626_x_at | v-ets erythroblastosis virus E26 oncogene like (avian) /// v-ets erythroblastosis virus E26 oncogene like (avian) | 2.64 |
| 214316_x_at | gb:AI378706 /DB_XREF=gi:4188559 /DB_XREF=tb91f09.x1 /CLONE=IMAGE:2061737 /FEA=EST /CNT=13 /TID=Hs.16488.3 /TIER=Stack /STK=13 /UG=Hs.16488 /LL=811 /UG_GENE=CALR /UG_TITLE=calreticulin | 2.64 |
| 220657_at | kelch-like 11 (Drosophila) | 2.61 |
| 206490_at | discs, large (Drosophila) homolog-associated protein 1 | 2.6 |
| 208383_s_at | phosphoenolpyruvate carboxykinase 1 (soluble) | 2.59 |
| 214884_at | gb:AL033403 /DB_XREF=gi:3859054 /FEA=mRNA /CNT=15 /TID=Hs.89543.1 /TIER=ConsEnd /STK=0 /UG=Hs.89543 /LL=4168 /UG_GENE=MCF2 /UG_TITLE=MCF.2 cell line derived transforming sequence /DEF=Human DNA sequence from clone 88D7 on chromosome Xq25-26.3 Contains F9 (coagulation factor IX (plasma thromboplastic component, Christmas disease, haemophilia B)), dbl oncogene. EST, STS, GSS | 2.59 |
| 201506_at | transforming growth factor, beta-induced, 68kDa | 2.18 |
| 213979_s_at | Consensus includes gb:BF984434 /FEA=EST /DB_XREF=gi:12387246 /DB_XREF=est:602307971F1 /CLONE=IMAGE:4399313 /UG=Hs.239737 C-terminal binding protein 1 | 2.5 |
| 211253_x_at | peptide YY | 2.38 |
| 206879_s_at | neuregulin 2 | 2.33 |
| 208835_s_at | cisplatin resistance-associated overexpressed protein | 2.33 |
| 201506_at | transforming growth factor, beta-induced, 68kDa | 2.18 |

## Table 3B. Genes Down-regulated by Ro25-7386 in MCF-7

| probe set | gene | fold change |
|---|---|---|
| 202901_x_at | cathepsin S | -64.37 |
| 201367_s_at | zinc finger protein 36, C3H type-like 2 | -5.67 |
| 213606_s_at | Rho GDP dissociation inhibitor (GDI) alpha | -5.01 |
| 211136_s_at | cleft lip and palate associated transmembrane protein 1 | -4.59 |
| 204989_s_at | integrin, beta 4 | -4.51 |
| 213042_s_at | ATPase, Ca++ transporting, ubiquitous | -4.42 |
| 216971_s_at | plectin 1, intermediate filament binding protein 500kDa | -4.37 |
| 201167_x_at | Rho GDP dissociation inhibitor (GDI) alpha | -4.14 |
| 219529_at | chloride intracellular channel 3 | -3.97 |
| 218813_s_at | SH3-domain GRB2-like endophilin B2 | -3.93 |
| 211905_s_at | integrin, beta 4 | -3.87 |
| 211672_s_at | actin related protein 2/3 complex, subunit 4, 20kDa /// actin related protein 2/3 complex, subunit 4, 20kDa | -3.7 |
| 207521_s_at | ATPase, Ca++ transporting, ubiquitous | -3.44 |
| 213986_s_at | chromosome 19 open reading frame 6 | -3.43 |
| 207824_s_at | MYC-associated zinc finger protein (purine-binding transcription factor) | -3.42 |
| 203085_s_at | transforming growth factor, beta 1 (Camurati-Engelmann disease) | -3.34 |
| 203953_s_at | claudin 3 | -3.26 |
| 211019_s_at | lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase) | -3.22 |
| 209872_s_at | plakophilin 3 | -3.2 |
| 214326_x_at | jun D proto-oncogene | -3.14 |
| 208677_s_at | basigin (OK blood group) | -3.12 |
| 201245_s_at | OTU domain, ubiquitin aldehyde binding 1 | -3.08 |
| 203751_x_at | jun D proto-oncogene | -3.08 |
| 203370_s_at | PDZ and LIM domain 7 (enigma) | -3.05 |
| 203028_s_at | cytochrome b-245, alpha polypeptide | -3.02 |
| 210954_s_at | KIAA0669 gene product | -2.99 |
| 211823_s_at | paxillin | -2.97 |
| 200968_s_at | peptidylprolyl isomerase B (cyclophilin B) | -2.93 |
| 205463_s_at | platelet-derived growth factor alpha polypeptide | -2.87 |
| 210317_s_at | tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide | -2.87 |
| 211300_s_at | tumor protein p53 (Li-Fraumeni syndrome) | -2.84 |
| 214251_s_at | nuclear mitotic apparatus protein 1 | -2.81 |
| 207722_s_at | BTB (POZ) domain containing 2 | -2.8 |
| 216969_s_at | kinesin family member 22 | -2.79 |
| 203809_s_at | v-akt murine thymoma viral oncogene homolog 2 | -2.76 |
| 218848_at | hypothetical protein MGC2655 | -2.73 |
| 212090_at | glutamate receptor, ionotropic, N-methyl D-asparate-associated protein 1 (glutamate binding) | -2.69 |
| 201373_at | plectin 1, intermediate filament binding protein 500kDa | -2.68 |
| 218302_at | presenilin enhancer 2 | -2.68 |
| 213887_s_at | polymerase (RNA) II (DNA directed) polypeptide E, 25kDa | -2.67 |
| 201369_s_at | zinc finger protein 36, C3H type-like 2 | -2.59 |
| 203392_s_at | C-terminal binding protein 1 | -2.5 |
| 200796_s_at | myeloid cell leukemia sequence 1 (BCL2-related) | -2.43 |
| 206665_s_at | BCL2-like 1 | -2.35 |

## Table 4A. Genes Up-regulated by LGD1069 in T47D

| probe set | gene | fold change |
|---|---|---|
| 215653_at | Consensus includes gb:AF339805.1 /DEF=Homo sapiens clone IMAGE:248602, mRNA sequence. /FEA=mRNA /DB_XREF=gi:13507343 /UG=Hs.326719 Homo sapiens clone IMAGE:248602, mRNA sequence | 4.74 |
| 206424_at | cytochrome P450, family 26, subfamily A, polypeptide 1 | 2.98 |
| 202481_at | dehydrogenase/reductase (SDR family) member 3 | 2.79 |
| 211689_s_at | transmembrane protease, serine 2 /// transmembrane protease, serine 2 | 2.61 |
| 213629_x_at | metallothionein 1F (functional) | 2.32 |
| 215924_at | Consensus includes gb:AK022102.1 /DEF=Homo sapiens cDNA FLJ12040 fis, clone HEMBB1001944. /FEA=mRNA /DB_XREF=gi:10433423 /UG=Hs.296687 Homo sapiens cDNA FLJ12040 fis, clone HEMBB1001944 | 2.32 |
| 208581_x_at | metallothionein 1X | 2.31 |
| 210827_s_at | E74-like factor 3 (ets domain transcription factor, epithelial-specific ) | 2.28 |
| 217165_x_at | metallothionein 1F (functional) | 2.23 |
| 204326_x_at | metallothionein 1X | 2.19 |
| 206461_x_at | metallothionein 1H | 2.15 |
| 204470_at | chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha) | 2.14 |
| 204745_x_at | metallothionein 1G | 2.13 |
| 217028_at | chemokine (C-X-C motif) receptor 4 | 2.04 |
| 212185_x_at | Consensus includes gb:NM_005953.1 /DEF=Homo sapiens metallothionein 2A (MT2A), mRNA. /FEA=CDS /GEN=MT2A /PROD=metallothionein 2A /DB_XREF=gi:5174763 /UG=Hs.118786 metallothionein 2A /FL=gb:NM_005953.1 | 2.02 |
| 211456_x_at | gb:AF333388.1 /DB_XREF=gi:13310411 /FEA=FLmRNA /CNT=1 /TID=Hs.326774.0 /TIER=FL /STK=0 /UG=Hs.326774 /DEF=Homo sapiens metallothionein 1H-like protein mRNA, complete cds. /PROD=metallothionein 1H-like protein /FL=gb:AF333388.1 | 2.01 |

## Table 4B. Genes Down-regulated by LGD1069 in T47D

| probe set | gene | fold change |
|---|---|---|
| 207437_at | neuro-oncological ventral antigen 1 | -3.04 |
| 210806_at | KIAA0998 | -2.33 |
| 202989_at | regulator of G-protein signalling 1 | -2.13 |

## Table 5A. Genes Up-regulated by LG100268 in T47D

| probe set | gene | fold change |
|---|---|---|
| 215653_at | Consensus includes gb:AF339805.1 /DEF=Homo sapiens clone IMAGE:248602, mRNA sequence. /FEA=mRNA /DB_XREF=gi:13507343 /UG=Hs.326719 Homo sapiens clone IMAGE:248602, mRNA sequence | 4.74 |
| 215924_at | Consensus includes gb:AK022102.1 /DEF=Homo sapiens cDNA FLJ12040 fis, clone HEMBB1001944. /FEA=mRNA /DB_XREF=gi:10433423 /UG=Hs.296687 Homo sapiens cDNA FLJ12040 fis, clone HEMBB1001944 | 2.87 |
| 204470_at | chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha) | 2.26 |

## Table 5B. Genes Down-regulated by LG100268 in T47D

| probe set | gene | fold change |
|---|---|---|
| 215655_at | Glutamate receptor, ionotropic, kainate 2 | -3.29 |
| 220327_at | colon carcinoma related protein | -2.94 |
| 213910_at | insulin-like growth factor binding protein 7 | -2.66 |
| 207145_at | growth differentiation factor 8 | -2.49 |
| 210806_at | KIAA0998 | -2.3 |

## Table 6A. Genes Up-regulated by LGD1069 in MDA-MB-231

| probe set | gene | fold change |
|---|---|---|
| 219948_x_at | hypothetical protein FLJ21934 | 232.43 |
| 209672_s_at | hypothetical protein FLJ20323 | 69.61 |
| 207750_at | gb:NM_018510.1 /DEF=Homo sapiens hypothetical protein PRO1866 (PRO1866), mRNA. /FEA=mRNA /GEN=PRO1866 /PROD=hypothetical protein PRO1866 /DB_XREF=gi:8924091 /UG=Hs.283031 hypothetical protein PRO1866 /FL=gb:AF119858.1 gb:NM_018510.1 | 30.5 |
| 203603_s_at | zinc finger homeobox 1b | 10.18 |
| 217698_at | Consensus includes gb:AV651668 /FEA=EST /DB_XREF=gi:9872682 /DB_XREF=est:AV651668 /CLONE=GLCCSC04 /UG=Hs.282480 ESTs | 10.11 |
| AFFX-r2-Ec-bioB-M_at | E. coli /GEN=bioB /DB_XREF=gb:J04423.1 /NOTE=SIF corresponding to nucleotides 2393-2682 of gb:J04423.1 /DEF=E.coli 7,8-diamino-pelargonic acid (bioA), biotin synthetase (bioB), 7-keto-8-amino-pelargonic acid synthetase (bioF), bioC protein, and dethiobiot | 9.76 |
| 205386_s_at | Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse) | 9.65 |
| 216119_s_at | chromosome 20 open reading frame 28 | 9.42 |
| AFFX-BioB-M_at | E. coli /GEN=bioB /DB_XREF=gb:J04423.1 /NOTE=SIF corresponding to nucleotides 2482-2739 of gb:J04423.1 /DEF=E.coli 7,8-diamino-pelargonic acid (bioA), biotin synthetase (bioB), 7-keto-8-amino-pelargonic acid synthetase (bioF), bioC protein, and dethiobiot | 9.32 |
| 209613_s_at | alcohol dehydrogenase IB (class I), beta polypeptide | 8.85 |
| AFFX-r2-Ec-bioB-3_at | E. coli /GEN=bioB /DB_XREF=gb:J04423.1 /NOTE=SIF corresponding to nucleotides 2772-3004 of gb:J04423.1 /DEF=E.coli 7,8-diamino-pelargonic acid (bioA), biotin synthetase (bioB), 7-keto-8-amino-pelargonic acid synthetase (bioF), bioC protein, and dethiobiot | 8.78 |
| 217194_at | Consensus includes gb:AB007970.1 /DEF=Homo sapiens mRNA, chromosome 1 specific transcript KIAA0501. /FEA=mRNA /DB_XREF=gi:3413945 /UG=Hs.223020 Homo sapiens mRNA, chromosome 1 specific transcript KIAA0501 | 7.08 |
| 205524_s_at | hyaluronan and proteoglycan link protein 1 | 7.06 |
| 215514_at | Consensus includes gb:AL080072.1 /DEF=Homo sapiens mRNA; cDNA DKFZp564M0616 (from clone DKFZp564M0616). /FEA=mRNA /DB_XREF=gi:5262482 /UG=Hs.21195 Homo sapiens mRNA; cDNA DKFZp564M0616 (from clone DKFZp564M0616) | 6.85 |
| 214774_x_at | trinucleotide repeat containing 9 | 6.7 |
| 215526_at | Consensus includes gb:AL050145.1 /DEF=Homo sapiens mRNA; cDNA DKFZp586C2020 (from clone DKFZp586C2020). /FEA=mRNA /DB_XREF=gi:4884356 /UG=Hs.225986 Homo sapiens mRNA; cDNA DKFZp586C2020 (from clone DKFZp586C2020) | 6.22 |
| 211091_s_at | neurofibromin 2 (bilateral acoustic neuroma) | 6.21 |
| 221959_at | hypothetical protein MGC39325 | 6.11 |
| 206863_x_at | gb:U76376.1 /DB_XREF=gi:1923234 /GEN=HRK /FEA=FLmRNA /CNT=9 /TID=Hs.87247.0 /TIER=ConsEnd /STK=0 /UG=Hs.87247 /LL=8739 /DEF=Homo sapiens activator of apoptosis Hrk (HRK) mRNA, complete cds. /PROD=activator of apoptosis Hrk /FL=gb:NM_003806.1 gb:U76376.1 | 6.09 |
| 206202_at | mesenchyme homeo box 2 (growth arrest-specific homeo box) | 5.75 |
| 205288_at | CDC14 cell division cycle 14 homolog A (S. cerevisiae) | 5.62 |
| 220931_at | hypothetical protein MGC5590 | 5.4 |
| 216795_at | CDNA: FLJ23194 fis, clone REC00490 | 5.29 |
| 206410_at | nuclear receptor subfamily 0, group B, member 2 | 5.23 |
| 207647_at | chromodomain protein, Y-linked, 1 /// chromodomain protein, Y-linked, 1B | 5.19 |
| 215112_x_at | MCF.2 cell line derived transforming sequence-like 2 | 5.11 |
| 216775_at | ubiquitin specific protease 53 | 4.9 |
| 220109_at | transferrin | 4.88 |
| 217132_at | Clone 24587 mRNA sequence | 4.86 |
| 216737_at | CDNA: FLJ20872 fis, clone ADKA02604 | 4.84 |
| 220036_s_at | lipocalin-interacting membrane receptor | 4.7 |
| AFFX-r2-Ec-bioD-3_at | E. coli /GEN=bioD /DB_XREF=gb:J04423.1 /NOTE=SIF corresponding to nucleotides 5312-5559 of gb:J04423.1, not 100% identical /DEF=E.coli 7,8-diamino-pelargonic acid (bioA), biotin synthetase (bioB), 7-keto-8-amino-pelargonic acid synthetase (bioF), bioC pro | 4.66 |
| 220564_at | chromosome 10 open reading frame 59 | 4.64 |
| 211611_s_at | tenascin XB /// tenascin XB /// cAMP responsive element binding protein-like 1 /// cAMP responsive element binding protein-like 1 | 4.61 |
| AFFX-BioDn- | E. coli /GEN=bioD /DB_XREF=gb:J04423.1 /NOTE=SIF corresponding to nucleotides 5286-5570 of gb:J04423.1, | 4.49 |

| | | |
|---|---|---|
| 3_at | not 100% identical /DEF=E.coli 7,8-diamino-pelargonic acid (bioA), biotin synthetase (bioB), 7-keto-8-amino-pelargonic acid synthetase (bioF), bioC pro | |
| 207272_at | zinc finger protein 80 (pT17) | 4.49 |
| 210690_at | killer cell lectin-like receptor subfamily C, member 4 | 4.47 |
| 216625_at | Consensus includes gb:AL050032.1 /DEF=Homo sapiens mRNA; cDNA DKFZp566F1224 (from clone DKFZp566F1224). /FEA=mRNA /DB_XREF=gi:4884272 /UG=Hs.306307 Homo sapiens mRNA; cDNA DKFZp566F1224 (from clone DKFZp566F1224) | 4.37 |
| 207245_at | UDP glycosyltransferase 2 family, polypeptide B17 | 4.35 |
| 208014_x_at | neuronal thread protein AD7c-NTP | 4.32 |
| 214767_s_at | heat shock protein, alpha-crystallin-related, B6 | 4.31 |
| 216697_at | Triple functional domain (PTPRF interacting) | 4.28 |
| 222341_x_at | Consensus includes gb:AW973235 /FEA=EST /DB_XREF=gi:8163081 /DB_XREF=est:EST385333 /UG=Hs.293697 ESTs | 4.27 |
| 207262_at | apolipoprotein F | 4.25 |
| 222320_at | Consensus includes gb:AW970584 /FEA=EST /DB_XREF=gi:8160429 /DB_XREF=est:EST382665 /UG=Hs.291033 ESTs | 4.14 |
| 206201_s_at | mesenchyme homeo box 2 (growth arrest-specific homeo box) | 4.06 |
| 208019_at | zinc finger protein 157 (HZF22) | 4.01 |
| 204991_s_at | neurofibromin 2 (bilateral acoustic neuroma) | 3.97 |
| 207607_at | achaete-scute complex-like 2 (Drosophila) | 3.88 |
| AFFX-r2-Ec-bioD-5_at | E. coli /GEN=bioD /DB_XREF=gb:J04423.1 /NOTE=SIF corresponding to nucleotides 5024-5244 of gb:J04423.1 /DEF=E.coli 7,8-diamino-pelargonic acid (bioA), biotin synthetase (bioB), 7-keto-8-amino-pelargonic acid synthetase (bioF), bioC protein, and dethiobiot | 3.83 |
| 211315_s_at | calcium channel, voltage-dependent, alpha 1G subunit | 3.78 |
| 205953_at | leucine-rich repeats and immunoglobulin-like domains 2 | 3.75 |
| 207781_s_at | zinc finger protein 6 (CMPX1) | 3.74 |
| 216068_at | Sodium- and chloride-activated ATP-sensitive potassium channel | 3.69 |
| 214899_at | hypothetical BC331191_1 | 3.59 |
| 208212_s_at | anaplastic lymphoma kinase (Ki-1) | 3.58 |

## Table 6B. Genes Down-regulated by LGD1069 in MDA-MB-231

| probe set | gene | fold change |
|---|---|---|
| 215117_at | recombination activating gene 2 | -60.45 |
| 217535_at | Consensus includes gb:AV720514 /FEA=EST /DB_XREF=gi:10817666 /DB_XREF=est:AV720514 /CLONE=GLCGSB09 /UG=Hs.282721 ESTs, Weakly similar to ALU7_HUMAN ALU SUBFAMILY SQ SEQUENCE CONTAMINATION WARNING ENTRY H.sapiens | -16.22 |
| 201691_s_at | tumor protein D52 | -16.09 |
| 207674_at | Fc fragment of IgA, receptor for | -6.54 |
| 215172_at | DKFZP566K0524 protein | -5.85 |
| 218541_s_at | chromosome 8 open reading frame 4 | -5.79 |
| 215350_at | spectrin repeat containing, nuclear envelope 1 | -5.69 |
| AFFX-HUMRGE/M10098_5_at | H. sapiens /GEN=18S rRNA /DB_XREF=gb:M10098.1 /NOTE=SIF corresponding to nucleotides 115-595 of gb:M10098.1 /DEF=Human 18S rRNA gene, complete. | -5.59 |
| 213652_at | Proprotein convertase subtilisin/kexin type 5 | -5.57 |
| 216050_at | Transcribed locus, moderately similar to NP_803425.1 DNA segment, Chr 19, Brigham & Women's Genetics 1357 expressed [Mus musculus] | -5.43 |
| 222342_at | Consensus includes gb:AW979196 /FEA=EST /DB_XREF=gi:8170484 /DB_XREF=est:EST391306 /UG=Hs.292713 ESTs, Moderately similar to ALU1_HUMAN ALU SUBFAMILY J SEQUENCE CONTAMINATION WARNING ENTRY H.sapiens | -5.41 |
| 205638_at | brain-specific angiogenesis inhibitor 3 | -5.04 |
| 217464_at | Consensus includes gb:L48784 /DEF=050 Homo sapiens cDNA /FEA=mRNA /DB_XREF=gi:1066715 /UG=Hs.182426 ribosomal protein S2 | -4.97 |
| 205848_at | growth arrest-specific 2 | -4.86 |
| 206588_at | deleted in azoospermia-like | -4.75 |
| 213826_s_at | Consensus includes gb:AA292281 /FEA=EST /DB_XREF=gi:1940261 /DB_XREF=est:zt51b03.s1 /CLONE=IMAGE:725837 /UG=Hs.181307 H3 histone, family 3A | -4.74 |
| 220432_s_at | cytochrome P450, family 39, subfamily A, polypeptide 1 | -4.48 |
| 209227_at | tumor suppressor candidate 3 | -4.41 |
| 211712_s_at | annexin A9 /// annexin A9 | -4.31 |
| AFFX-HUMRGE/M10098_M_at | H. sapiens /GEN=18S rRNA /DB_XREF=gb:M10098.1 /NOTE=SIF corresponding to nucleotides 688-1219 of gb:M10098.1 /DEF=Human 18S rRNA gene, complete. | -4.28 |
| AFFX-HUMRGE/M10098_3_at | signal recognition particle 68kDa | -4.2 |
| 202648_at | gb:BC000023.1 /DB_XREF=gi:12652562 /FEA=FLmRNA /CNT=966 /TID=Hs.298262.0 /TIER=ConsEnd /STK=0 /UG=Hs.298262 /LL=6223 /UG_GENE=RPS19 /DEF=Homo sapiens, ribosomal protein S19, clone MGC:1630, mRNA, complete cds. /PROD=ribosomal protein S19 /FL=gb:M81757.1 g | -4.15 |
| 207815_at | platelet factor 4 variant 1 | -4.15 |
| 205363_at | butyrobetaine (gamma), 2-oxoglutarate dioxygenase (gamma-butyrobetaine hydroxylase) 1 | -4.14 |
| 213856_at | CD47 antigen (Rh-related antigen, integrin-associated signal transducer) | -4.11 |
| 216087_at | MRNA full length insert cDNA clone EUROIMAGE 117929 | -4.11 |
| 211264_at | glutamate decarboxylase 2 (pancreatic islets and brain, 65kDa) | -4.03 |
| 220771_at | melanoma antigen | -3.83 |
| 220474_at | solute carrier family 25 (mitochondrial oxodicarboxylate carrier), member 21 | -3.81 |
| 220281_at | solute carrier family 12 (sodium/potassium/chloride transporters), member 1 | -3.8 |
| 217524_x_at | Consensus includes gb:AA018923 /FEA=EST /DB_XREF=gi:1482314 /DB_XREF=est:ze58d03.s1 /CLONE=IMAGE:363173 /UG=Hs.261204 ESTs | -3.72 |
| 211776_s_at | erythrocyte membrane protein band 4.1-like 3 /// erythrocyte membrane protein band 4.1-like 3 | -3.69 |
| 212681_at | erythrocyte membrane protein band 4.1-like 3 | -3.69 |
| 217333_at | Consensus includes gb:AL031903 /DEF=Human DNA sequence from clone 1032F13 on chromosome Xq25-26.3. Contains a pseudogene similar to Keratin 18 (KRT18, Cytokeratin 18) and ESTs /FEA=CDS /DB_XREF=gi:3766260 /UG=Hs.247763 Human DNA sequence from clone 1032F1 | -3.69 |
| 210721_s_at | p21(CDKN1A)-activated kinase 7 | -3.63 |
| 210327_s_at | alanine-glyoxylate aminotransferase (oxalosis I; hyperoxaluria I; glycolicaciduria; serine-pyruvate aminotransferase) | -3.57 |
| 206265_s_at | glycosylphosphatidylinositol specific phospholipase D1 | -3.54 |
| 205847_at | protease, serine, 22 | -3.52 |
| 202901_x_at | cathepsin S | -3.42 |
| 204681_s_at | Rap guanine nucleotide exchange factor (GEF) 5 | -3.35 |
| 222227_at | Zinc finger protein 236 | -3.35 |
| 207465_at | PRO0628 protein | -3.34 |

**Table 7A. Genes Up-regulated by LG100268 in MDA-MB-231**

| probe set | gene | fold change |
|---|---|---|
| 219948_x_at | hypothetical protein FLJ21934 | 88.95 |
| 207750_at | gb:NM_018510.1 /DEF=Homo sapiens hypothetical protein PRO1866 (PRO1866), mRNA. /FEA=mRNA /GEN=PRO1866 /PROD=hypothetical protein PRO1866 /DB_XREF=gi:8924091 /UG=Hs.283031 hypothetical protein PRO1866 /FL=gb:AF119858.1 gb:NM_018510.1 | 26.42 |
| 209672_s_at | hypothetical protein FLJ20323 | 14.63 |
| 215514_at | Consensus includes gb:AL080072.1 /DEF=Homo sapiens mRNA; cDNA DKFZp564M0616 (from clone DKFZp564M0616). /FEA=mRNA /DB_XREF=gi:5262482 /UG=Hs.21195 Homo sapiens mRNA; cDNA DKFZp564M0616 (from clone DKFZp564M0616) | 9.11 |
| 215309_at | Transcribed locus, weakly similar to XP_092995.4 zinc finger protein 21 (KOX 14) [Homo sapiens] | 8.12 |
| 214774_x_at | trinucleotide repeat containing 9 | 7.58 |
| 203603_s_at | zinc finger homeobox 1b | 5.77 |
| 205386_s_at | Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse) | 5.2 |
| 205419_at | Epstein-Barr virus induced gene 2 (lymphocyte-specific G protein-coupled receptor) | 4.18 |
| 216978_x_at | Consensus includes gb:U50277.1 /DEF=Human breast cancer suppressor element Ishmael Upper CP1 mRNA, partial cds. /FEA=mRNA /PROD=suppressor element Ishmael Upper CP1 /DB_XREF=gi:1224126 /UG=Hs.121485 Human breast cancer suppressor element Ishmael Upper CP | 3.93 |
| 220931_at | hypothetical protein MGC5590 | 3.81 |
| 219995_s_at | hypothetical protein FLJ13841 | 3.77 |
| 208076_at | histone 1, H4d | 3.6 |
| 214255_at | ATPase, Class V, type 10A | 3.55 |
| 207987_s_at | gonadotropin-releasing hormone 1 (luteinizing-releasing hormone) | 3.52 |
| 205651_x_at | Rap guanine nucleotide exchange factor (GEF) 4 | 3.46 |
| 220401_at | hypothetical protein FLJ21369 | 3.39 |
| 207241_at | chromosome 4 open reading frame 6 | 3.35 |
| 215623_x_at | SMC4 structural maintenance of chromosomes 4-like 1 (yeast) | 3.17 |
| 216119_s_at | chromosome 20 open reading frame 28 | 3.13 |
| 217194_at | Consensus includes gb:AB007970.1 /DEF=Homo sapiens mRNA, chromosome 1 specific transcript KIAA0501. /FEA=mRNA /DB_XREF=gi:3413945 /UG=Hs.223020 Homo sapiens mRNA, chromosome 1 specific transcript KIAA0501 | 3.1 |
| 206381_at | sodium channel, voltage-gated, type II, alpha 2 | 3.09 |
| 212182_at | nudix (nucleoside diphosphate linked moiety X)-type motif 4 | 2.98 |
| 215112_x_at | MCF.2 cell line derived transforming sequence-like 2 | 2.94 |
| 213747_at | Consensus includes gb:AA047234 /FEA=EST /DB_XREF=gi:1525134 /DB_XREF=est:zf50b09.s1 /CLONE=IMAGE:380345 /UG=Hs.223014 antizyme inhibitor | 2.84 |
| 221683_s_at | centrosome protein cep290 | 2.8 |
| 211611_s_at | tenascin XB /// tenascin XB /// cAMP responsive element binding protein-like 1 /// cAMP responsive element binding protein-like 1 | 2.74 |
| 205421_at | solute carrier family 22 (extraneuronal monoamine transporter), member 3 | 2.66 |
| 213764_s_at | microfibrillar associated protein 5 | 2.62 |
| 217505_at | hypothetical protein MGC22679 | 2.61 |
| 222320_at | Consensus includes gb:AW970584 /FEA=EST /DB_XREF=gi:8160429 /DB_XREF=est:EST382665 /UG=Hs.291033 ESTs | 2.61 |
| 216466_at | Neuron navigator 3 | 2.59 |
| AFFX-r2-Ec-bioB-M_at | E. coli /GEN=bioB /DB_XREF=gb:J04423.1 /NOTE=SIF corresponding to nucleotides 2393-2682 of gb:J04423.1 /DEF=E.coli 7,8-diamino-pelargonic acid (bioA), biotin synthetase (bioB), 7-keto-8-amino-pelargonic acid synthetase (bioF), bioC protein, and dethiobiot | 2.55 |
| 216775_at | ubiquitin specific protease 53 | 2.54 |
| 206201_s_at | mesenchyme homeo box 2 (growth arrest-specific homeo box) | 2.53 |
| AFFX-BioDn-5_at | E. coli /GEN=bioD /DB_XREF=gb:J04423.1 /NOTE=SIF corresponding to nucleotides 4980-5256 of gb:J04423.1, not 100% identical /DEF=E.coli 7,8-diamino-pelargonic acid (bioA), biotin synthetase (bioB), 7-keto-8-amino-pelargonic acid synthetase (bioF), bioC pro | 2.48 |
| 216894_x_at | cyclin-dependent kinase inhibitor 1C (p57, Kip2) | 2.46 |
| 208019_at | zinc finger protein 157 (HZF22) | 2.45 |
| 215803_at | hypothetical protein FLJ10178 | 2.44 |
| 222320_at | CDNA: FLJ23194 fis, clone REC00490 | 2.44 |

## Table 7B. Genes Down-regulated by LG100268 in MDA-MB-231

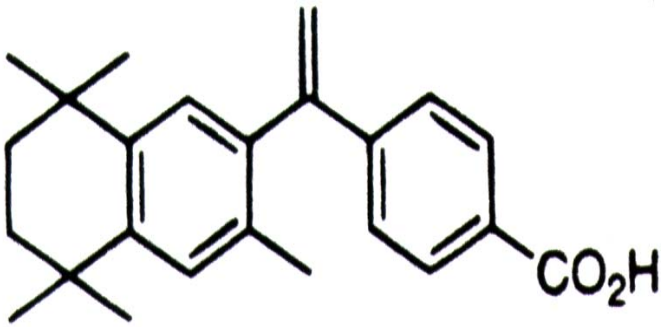
| probe set | gene | fold change |
|---|---|---|
| 217237_at | Zinc finger protein 423 | -78.6 |
| 215014_at | Consensus includes gb:AL512727.1 /DEF=Homo sapiens mRNA; cDNA DKFZp547P042 (from clone DKFZp547P042). /FEA=mRNA /DB_XREF=gi:12224870 /UG=Hs.232127 Homo sapiens mRNA; cDNA DKFZp547P042 (from clone DKFZp547P042) | -17.74 |
| 213753_x_at | eukaryotic translation initiation factor 5A | -7.65 |
| 212382_at | Transcription factor 4 | -5.74 |
| AFFX-HUMRGE/M10098_5_at | H. sapiens /GEN=18S rRNA /DB_XREF=gb:M10098.1 /NOTE=SIF corresponding to nucleotides 115-595 of gb:M10098.1 /DEF=Human 18S rRNA gene, complete. | -5.58 |
| 211712_s_at | annexin A9 /// annexin A9 | -5.49 |
| 209227_at | tumor suppressor candidate 3 | -5.11 |
| 216917_s_at | synaptonemal complex protein 1 | -4.82 |
| AFFX-HUMRGE/M10098_M_at | H. sapiens /GEN=18S rRNA /DB_XREF=gb:M10098.1 /NOTE=SIF corresponding to nucleotides 688-1219 of gb:M10098.1 /DEF=Human 18S rRNA gene, complete. | -4.31 |
| 210697_at | zinc finger protein 257 | -4.11 |
| 215013_s_at | ubiquitin specific protease 34 | -3.97 |
| 209657_s_at | heat shock transcription factor 2 | -3.96 |
| 221009_s_at | angiopoietin-like 4 | -3.9 |
| 205612_at | multimerin 1 | -3.79 |
| 207613_s_at | calcium/calmodulin-dependent protein kinase (CaM kinase) II alpha | -3.55 |
| 37232_at | KIAA0586 | -3.38 |
| AFFX-HUMRGE/M10098_3_at | signal recognition particle 68kDa | -3.37 |
| 204422_s_at | fibroblast growth factor 2 (basic) | -3.33 |
| 220638_s_at | Cas-Br-M (murine) ecotropic retroviral transforming sequence c | -3.32 |
| 208098_at | olfactory receptor, family 12, subfamily D, member 3 /// olfactory receptor, family 12, subfamily D, member 3 /// olfactory receptor, family 5, subfamily V, member 1 /// olfactory receptor, family 5, subfamily V, member 1 | -3.29 |
| 213826_s_at | Consensus includes gb:AA292281 /FEA=EST /DB_XREF=gi:1940261 /DB_XREF=est:zt51b03.s1 /CLONE=IMAGE:725837 /UG=Hs.181307 H3 histone, family 3A | -3.25 |
| 208453_s_at | X-prolyl aminopeptidase (aminopeptidase P) 1, soluble | -3.2 |
| 207485_x_at | butyrophilin, subfamily 3, member A1 | -3.18 |
| 211032_at | COBL-like 1 /// COBL-like 1 | -3.11 |
| 220619_at | chromodomain helicase DNA binding protein 7 | -3.04 |
| 209318_x_at | pleiomorphic adenoma gene-like 1 | -3 |
| 201547_at | Jumonji, AT rich interactive domain 1B (RBP2-like) | -2.99 |
| 206996_x_at | calcium channel, voltage-dependent, beta 1 subunit | -2.98 |
| 220114_s_at | stabilin 2 | -2.95 |
| 216709_at | Hypothetical gene supported by BC013370; BC034583 | -2.93 |
| 203555_at | protein tyrosine phosphatase, non-receptor type 18 (brain-derived) | -2.92 |
| 213267_at | KIAA1117 | -2.91 |
| 201122_x_at | eukaryotic translation initiation factor 5A | -2.89 |
| 213495_s_at | gb:AW166067 /DB_XREF=gi:6397592 /DB_XREF=xf44g10.x1 /CLONE=IMAGE:2620962 /FEA=EST /CNT=75 /TID=Hs.98614.2 /TIER=Stack /STK=51 /UG=Hs.98614 /LL=6238 /UG_GENE=RRBP1 /UG_TITLE=ribosome binding protein 1 (dog 180kD homolog) | -2.89 |
| 220301_at | chromosome 18 open reading frame 14 | -2.88 |
| 214837_at | albumin | -2.85 |
| 209700_x_at | phosphodiesterase 4D interacting protein (myomegalin) | -2.84 |
| 216805_at | Transcribed locus, moderately similar to XP_375099.1 hypothetical protein LOC283585 [Homo sapiens] | -2.84 |
| 221671_x_at | immunoglobulin kappa constant | -2.79 |
| 214001_x_at | gb:AW302047 /DB_XREF=gi:6711724 /DB_XREF=xr52f08.x1 /CLONE=IMAGE:2763783 /FEA=EST /CNT=24 /TID=Hs.76230.2 /TIER=Stack /STK=20 /UG=Hs.76230 /LL=6204 /UG_GENE=RPS10 /UG_TITLE=ribosomal protein S10 | -2.72 |
| 210047_at | solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2 | -2.69 |
| 208367_x_at | cytochrome P450, family 3, subfamily A, polypeptide 4 | -2.66 |
| 219252_s_at | family with sequence similarity 51, member A1 | -2.65 |
| 205827_at | cholecystokinin | -2.63 |

## Table 8. Forward and reverse primers for amplification of targeted genes with real-time RT-PCR

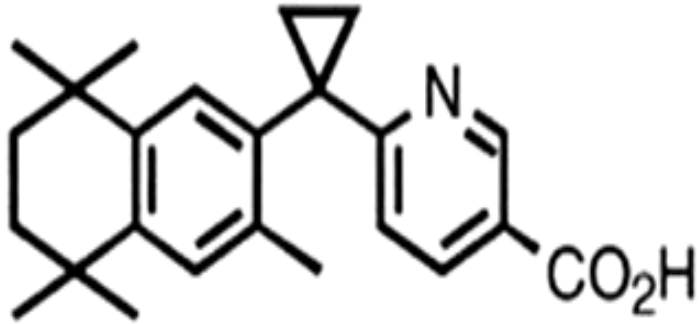
| Target genes | Forward primers | Reverse primers |
|---|---|---|
| BAX | 5'-TGGAGCTGCAGAGGATGATTG-3' | 5'-GAAGTTGCCGTCAGAAAACATG-3' |
| E-cadherin = CDH1 | 5'-CACTGCCAACTGGCTGGAG-3' | 5'-GGGTTAGCTCAGCAGTAAAG-3' |
| FOXO3A=FKHRL1 | 5'-TCAATCAGAACTTGCTCCACCA-3' | 5'-GGACTCACTCAAGCCCATGTTG-3' |
| Integrin alpha 6 | 5- TTTCCCGTTTCTTTCTTGAGTTGT-3' | 5'-TGGAAAAGGTAACTTGTGAGCCA-3' |
| Integrin beta 4 | 5-TTCCAAATCACAGAGGAGAC-3 | 5-CTTGAGGTTGTCCAGATCAT-3' |
| PXN = paxillin | 5'-TGGCTTCGCTGTCGGATTTC-3' | 5'GTCAAGGGCTGTCACCACTTTATC-3' |
| PTEN | 5'-AGAGCGTGCAGATAATGACAAG-3' | 5'-GGATCAGAGTCAGTGGTGTCAG-3' |
| STAT | 5'-CTGCTGCGGTTCAGTGAGAG-3' | 5'-CCAAGTGAAAGTGACCCCTCC-3' |
| Collagen type VI alpha 3 | 5'-CTGGGCAGACATACCATGTG-3' | 5'-GCAAGTTCCTTCGTCTTTCG-3' |

# SUPPORTING DATA

# Molecular Structure of LGD1069 and LG100268




**LGD1069**




**LG100268**

**Figure 1**

# Expression of RXR alpha in breast cells (western blot analysis) – normal (HMEC) and breast cancer cells

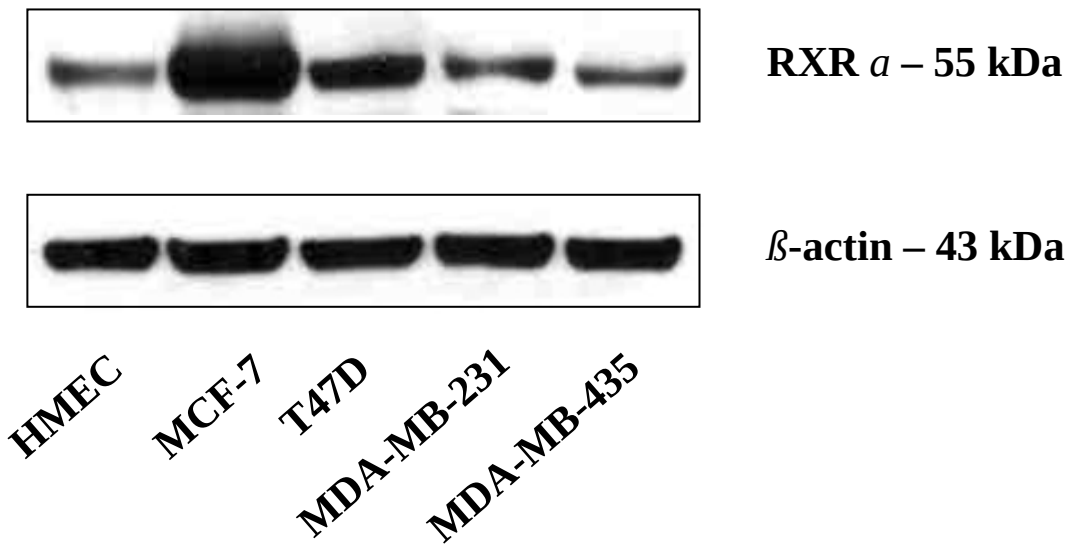
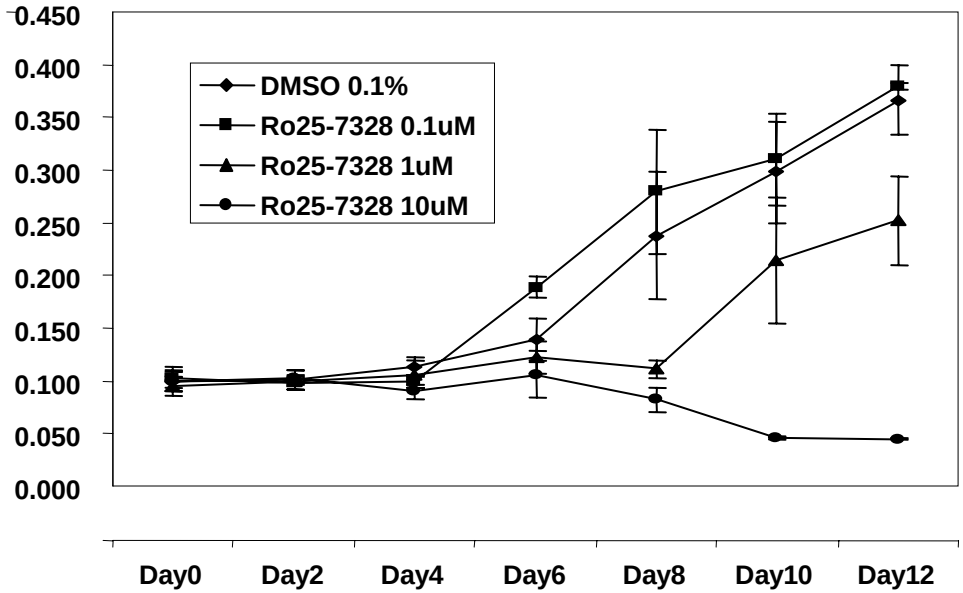
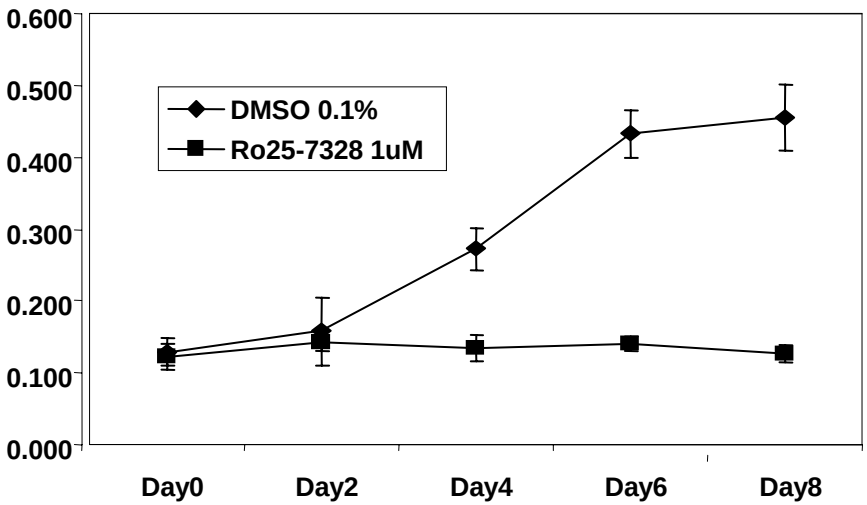



**Figure 2**

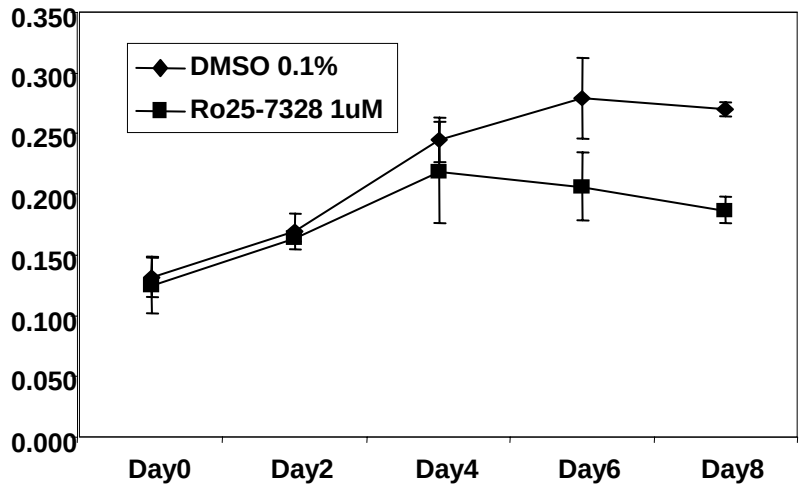
# Cell Proliferation Assay with RXR alpha ligand (Ro25-7386)

HMEC




T47D




MCF-7



**Figure 3**

# The effect of LGD1069 and LG100268 on the growth of HMEC

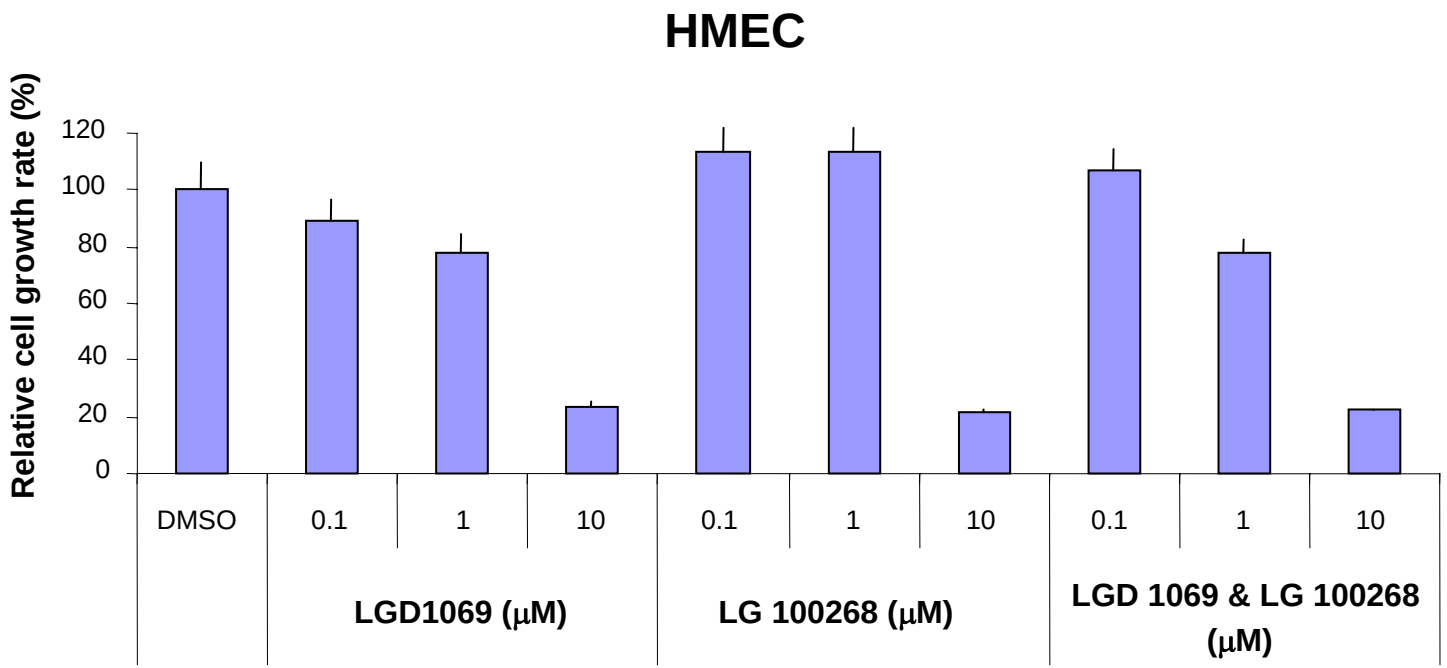



**Figure 4**

# The effect of LGD1069 and LG100268 on the growth of MCF-7 and T47D

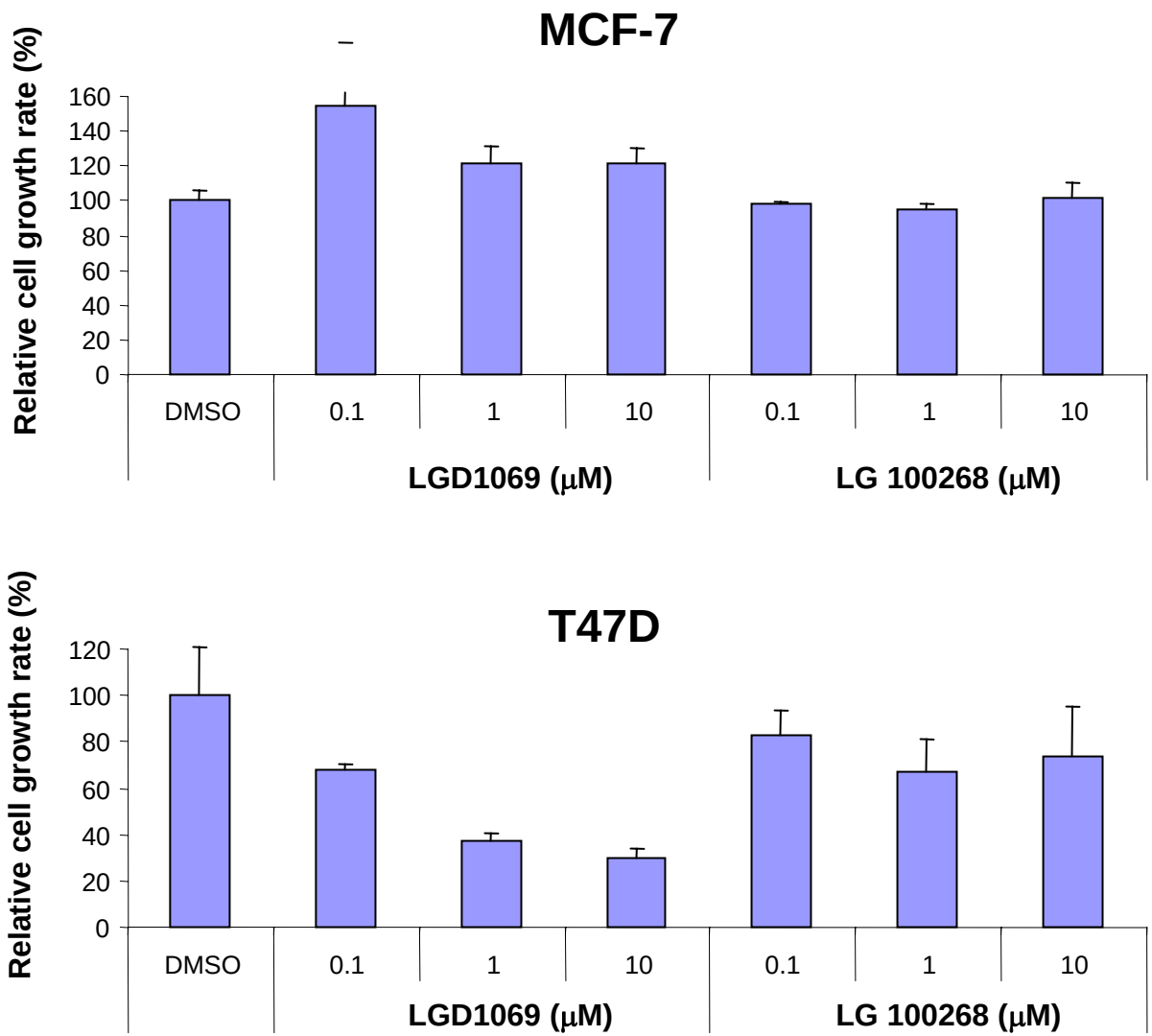



**Figure 5**

# The effect of LGD1069 and LG100268 on the growth of MDA-MB-231 and MDA-MB-435

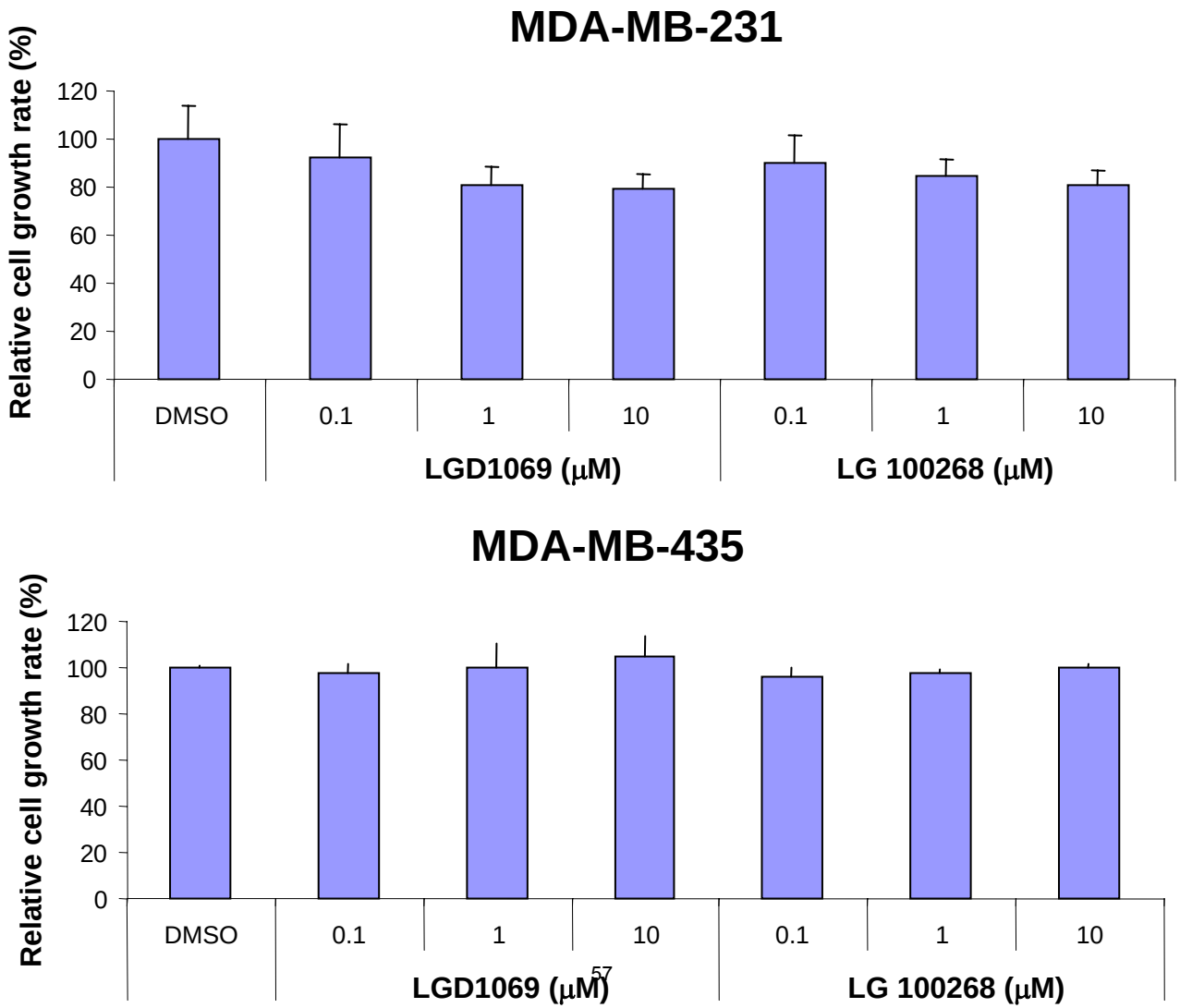



**Figure 6**

## Cell Growth Suppressive Activity

| | HMEC | MCF-7 | T47D | MDA-MB-231 |
|---|---|---|---|---|
| Ro25-7386 | ● | ● | ● | X |
| LGD1069 | ● | X | ● | ● |
| LG100268 | ● | X | X | ● |

## Affymetrix Microarray Data

| | HMEC | MCF-7 | T47D | MDA-MB-231 |
|---|---|---|---|---|
| Ro25-7386 | ● | ● | ● | X |
| LGD1069 | X | X | ● | ● |
| LG100268 | X | X | X | ● |



**Figure 7**

# mRNA levels of RXRα-regulated genes in HMEC (measurement by real-time RT-PCR)

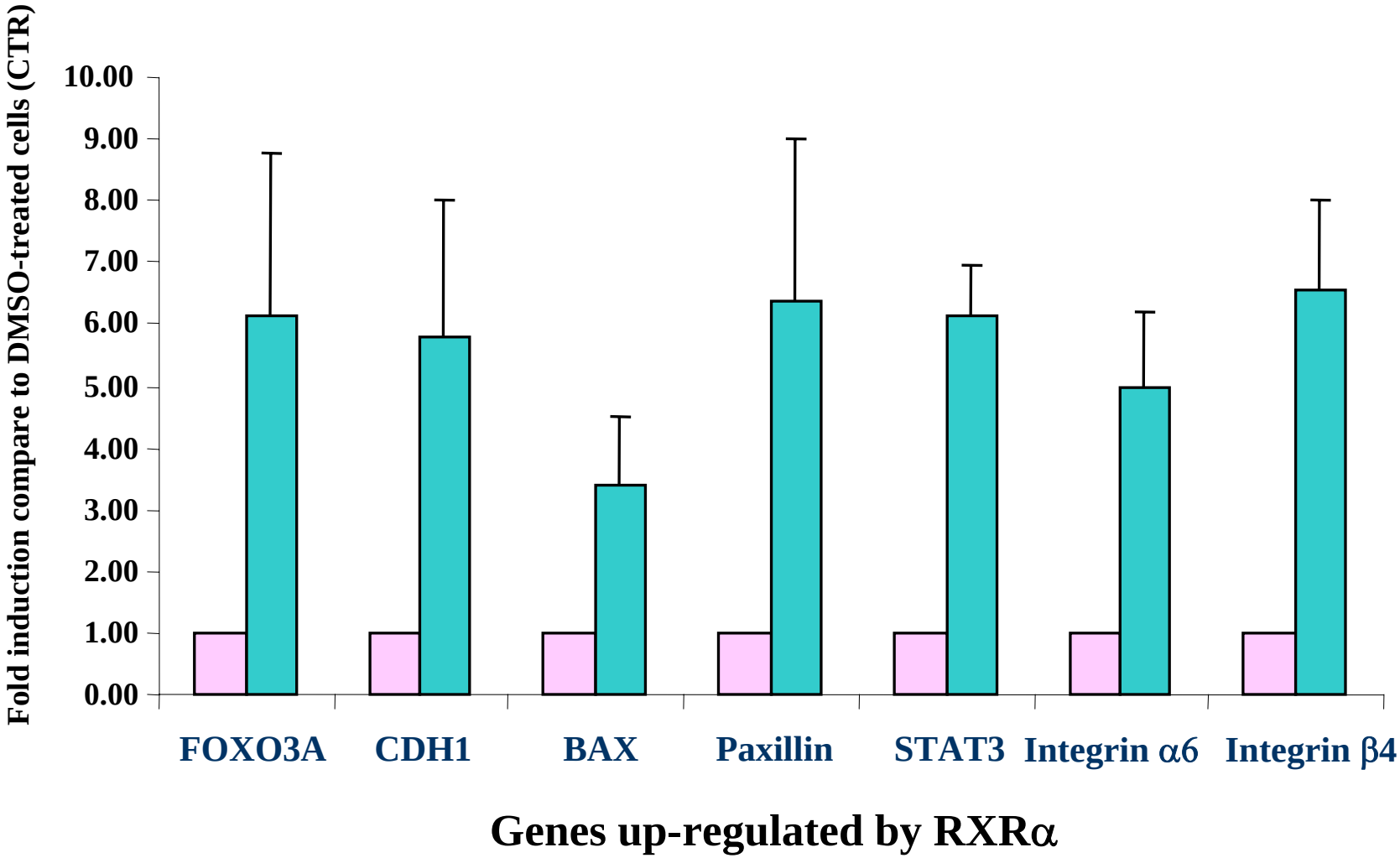





**Figure 8**

# Protein levels of RXRα-regulated genes in HMEC (measurement by Western blot analysis)

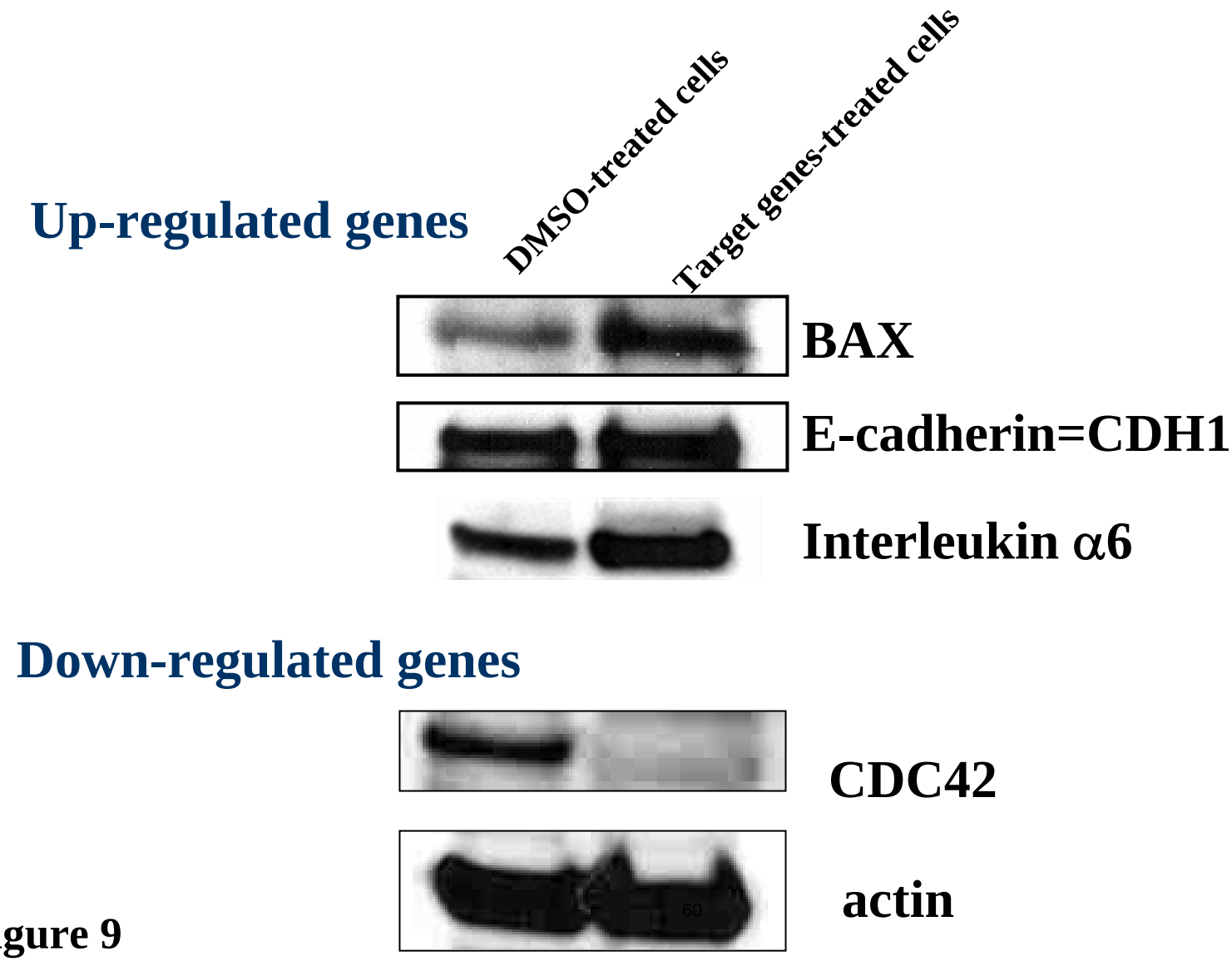



Figure 9